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Patentanmeldung Nr. **Patent application No.** **Demande de brevet n°**

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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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R C van Dijk

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Methods and system for the identification and characterization of peptides and their functional relationships by use of measures of correlation

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Our Ref.: 412-4

METHODS AND SYSTEM FOR THE IDENTIFICATION AND CHARACTERIZATION OF PEPTIDES AND THEIR FUNCTIONAL RELATIONSHIPS BY USE OF MEASURES OF CORRELATION

Technical Field of the Invention

The invention relates to the field of high-throughput analysis of samples with peptide content and especially computer implemented methods and a system implementing these methods for identifying and characterizing peptides and their functional relationships by use of measures of correlation.

Background of the Invention

The success of the Human Genome Project in mapping the human genetic code offers astonishing potential for medical research. A prerequisite for using this information, however, is the identification of gene products, especially proteins and peptides. Peptides are the family of molecules formed from the linking, in a defined order, of various amino acids. The link between one amino acid residue and the next is an amide bond, and is sometimes referred to as a peptide bond. Peptides occur in nature and are responsible for a variety of functions, many of which are not understood. They differ from proteins, which are also long chains of amino acids, by virtue of their size.

Parallel to the world-wide efforts in Genomics, a variety of discovery technologies have been developed for analyzing samples with peptide content. Just as Genomics focuses on decoding the human genome, these technologies strive for an comprehensive analysis of the myriad of biologically relevant proteins and peptides with a molecular mass between about 0.5 and 20 kDa, among which insulin is a prominent example.

Profiling of peptides and proteins of human body fluids and tissues by mass spectrometry reveals a large number of peptide signals. Such high-throughput analytical processes demand highly sophisticated bioinformatic approaches to understand and analyze biological and pharmaceutical coherences in huge sets of data.

Conventional computer implemented methods for assisting the mass spectrometric identification of peptides and small proteins interpret the spectra and generate proposals for the identity of the candidate peptide signal by determining the differences of masses of the fragments in one spectra and assigning these differences to missing amino acids. A string of missing amino acids is then composed to a proposed amino acid sequence that is thereafter queried in a huge database containing tens of thousands of the known protein sequences, such as the Swiss-Prot database. However, if the analyzed peptide or protein is not abundant and/or in a complex mixture, such an approach turns out to be not very effective and, thus, time consuming concentration or fractionation steps of the sample have to be performed.

More sophisticated approaches take the knowledge of a known sequence in a spectrum into consideration. Here, proteolytic digests of the known sequence are proposed "in silico", and a hypothetic resulting spectrum is then correlated with the actually measured one. However, these approaches are successful only if the sources of the spectra contain only a few different analytes, as their fragment signals alter the calculations and lower the correlation coefficients of the hypothesized calculated spectra with the actually measured one. If many possible protein precursors exist for a given peptide, then creating such a hypothetic spectrum for each unknown peptide and each possible precursor, the correlation process of hypothetic and measured spectra often turn out to be quite laborious and at times even unsuccessful.

Eng et al (*Journ. Am. Soc. Mass Spectrom.* 5, 976-989, 1994) for instance describe a statistical scorer for tandem mass spectrometry, that relies on cross-correlating experimental spectra with predicted spectra of peptides from a database (Havilio et al, *Anal. Chem.* 75[3], 435-444, 2003). No additional information (e.g. enzyme specificity used to create the peptides) about the peptide except the mass of the peptide is used. In a first step the tandem mass spectrometry data is reduced, whereby all but the most abundant signals are removed. In a second step protein sequences are queried from a database for combinations of amino acids that match the mass of the peptide, wherein the search algorithm only considers mass changes typical for a post-translational modification at every occurrence of the modification site. In a third step, the preliminary matches are scored by summing the number of fragmented ions that match the ions observed in the spectrum. Immonium ions are considered if the sequence contains the amino acids Tyrosine, Tryptophan, Methionine or Phenylalanine. This and the sum of fragments are being taken into account in the scoring function. Finally, a spectrum is reconstructed from the putative amino acid sequences and the highest scoring predictions are assessed by a cross-correlational analysis. The cross-correlation function measures the coherence of the reconstructed and the measured spectrum signals by, in effect, translating one signal across the other. Well-known applications such as SEQUEST and Sonar make use of this approach. However, a disadvantage of this approach is that the peak intensity strongly depends on the ion type, the ion mass and other experimental parameters and that many factors are not fully understood yet that contribute to peptide fragmentation.

Perkins et al (*Electrophoresis* 20[18], 3551-3567, 1999) describe a statistical scorer that evaluates the probability of finding a collection of detected fragments in a protein database (Havilio et al, *Anal. Chem.* 75[3], 435-444, 2003). Applications such as Mascot, MOWSE, Protocall are based on this approach. However, a disadvantage of this approach is that the signal intensities of the measured spectra are not being considered for the data analysis.

Weinberger et al (United States Patent Application 2002/0182649) describe essentially two approaches. In the first approach a protein candidate is identified by providing the mass spectrum to a protein database mining tool which identifies at least one protein candidate for the test protein in the database based on a closeness-of-fit measure between the mass spectrum and the theoretically calculated mass spectra of proteins in the database. In the second approach, the protein candidate is directly sequenced using mass spectrometry. In this method the unknown peptide is directly fragmented during mass spectrometry and the masses of the generated fragments are determined by mass spectrometry and are used to calculate the sequence of the unknown peptide.

The approaches according to Eng et al and Weinberger et al have in common that a closeness-of-fit analysis or a cross-correlation is performed over all signals of two spectra, i.e. the measured spectrum and the predicted spectrum. A fundamental disadvantage of these methods is that they rely on predicted mass spectra.

Thus, all of the above approaches have their disadvantages in that at times they turn out to be not very effective, quite laborious, time consuming and often unsuccessful.

There is thus a need for methods for analyzing samples with peptide content and a system implementing these methods overcoming or at least mitigating the disadvantages associated with the prior art.

Summary of the Invention

The following methods according to the present invention are based upon the concept of Correlation Associated Networks and peptide topologies as will be apparent from the detailed description in the sections further below.

According to the present invention a method based on CANs is provided for providing a representative, non-redundant overview of the peptide content of a sample type by analyzing a plurality of samples using its peptide topology, wherein the method comprises the steps of providing a respective mass spectrum for each sample of said plurality of samples, wherein signal intensity peaks correspond to potential peptides, computing the measures of correlation between the signal intensities of said potential peptides, grouping potential peptides together, which exhibit a degree of correlation among each other above a certain threshold, thereby providing a plurality of correlation associated networks of potential peptides, and assigning one representative potential peptide out of each correlation associated network as a representative peptide to said correlation associated network of said sample type.

Furthermore, a method based on CANs is provided for predicting the sequence of peptides using the peptide topology of a plurality of samples containing a peptide having a known precursor, wherein the method comprises the steps of providing a respective mass spectrum for each sample of said plurality of samples, wherein signal intensity peaks correspond to potential peptides, identifying said peptide having a known precursor using the mass of said peptide, wherein the sequence of the known precursor is known, computing the measures of correlation between the signal intensity of said peptide having a known precursor and the signal intensities of the other potential peptides, selecting potential peptides, which exhibit a degree of correlation with said peptide having a known precursor above a certain threshold, and predicting the sequence of the potential peptides by matching masses of putative fragments of the sequence of the known precursor with the masses of the potential peptides correlating with said peptide having a known precursor.

Still furthermore, a method based on CANs is provided for predicting the sequence of peptides using the peptide topology of a plurality of samples containing a peptide with a known sequence, wherein the method comprises the steps of providing a respective mass spectrum for each sample of said plurality of samples, wherein signal intensity peaks correspond to potential peptides, identifying a peptide with a known sequence using its mass, computing the measures of correlation between the signal intensity of said known peptide and the signal intensities of the potential peptides, selecting potential peptides, which exhibit a degree of correlation with the known peptide above a certain threshold, computing the mass differences between each of the potential peptides and the known peptide, and predicting the sequence and/or the biologically, chemically or physically modified sequence of the potential peptides by using data about mass differences caused by biological, chemical or physical processes matching the mass differences determined in the previous step.

Yet still furthermore, a method based on CANs is provided for identifying peptides suitable to be used as marker panels using the peptide topology of a plurality of samples taken from at least two different experimental groups representing a status A and a status B, wherein the method comprises the steps of providing a respective mass spectrum for each sample of said plurality of samples, wherein signal intensity peaks correspond to potential peptides, computing the measures of correlation between the signal intensities of said potential peptides for each plurality of samples within each experimental group separately, and selecting pairs of potential peptides, which exhibit a difference in the degree of correlation between the different experimental groups above a certain threshold, thereby providing peptides which are suitable to be used as marker panels for diagnostic purposes to distinguish between status A and status B.

Yet still furthermore, a method based on CANs is provided for identifying peptides suitable to be used as marker panels using the peptide topology of a plurality of samples taken from at least two different experimental groups representing a status A and a status B, wherein the method comprises the steps of providing a respective mass spectrum for each sample of said plurality of samples, wherein signal intensity peaks correspond to potential peptides, selecting potential peptides correlating with a parameter being representative of status A or status B, computing the measures of correlation between the signal intensities of said selected potential peptides for each plurality of samples, and selecting pairs of potential peptides which exhibit no correlation of their respective signal intensities above a certain threshold, thereby providing potential peptides which are suitable to be used as complementing peptides in a marker panel for diagnostic purposes to distinguish between status A and status B.

Finally, a method based on CANs is provided for identifying peptides suitable as a surrogate for a known peptide using the peptide topology of a plurality of samples, wherein the method comprises the steps of providing a respective mass spectrum for each sample of said plurality of samples, wherein signal intensity peaks correspond to potential peptides, computing the measures of correlation between the signal intensity of said known peptide and the signal intensities of potential peptides, and selecting potential peptides, which exhibit a degree of correlation with said known peptide above a certain threshold, thereby providing potential peptides suitable as a surrogate for said known peptide.

Preferred embodiments of the present invention are disclosed in the dependent claims.

Brief Description of the Drawings

FIGURE 1 shows schematically the hardware components and software modules according to the present invention, their interfaces as well as the flow of information between the hardware components and the software modules.

FIGURE 2 shows an averaged Peptide Mass Fingerprint of cerebrospinal fluid (CSF) samples from several patients. Each of the 96 chromatographic fractions of each sample is analyzed by MALDI-ToF-mass spectrometry and all 96 mass spectra generated from one sample are visualized as a "2-D gel-like picture", wherein the x- and y-axis correspond to the mass-to-charge ratio (m/z) and the chromatographic fraction (F), respectively. The bars represent peptide peaks, wherein the color intensity represents the mass spectrometric signal intensity. Some identified peptides including amino acid numbers are identified in this map.

FIGURE 3 shows a diagram exemplifying the correlational behavior of functionally related peptides. Four traces of spectra from four different samples are focused on the signals of a human osteopontin peptide being comprised of the amino acids 249-314 of human osteopontin (m/z = 7653.6 Da) and its phosphorylated derivatives, carrying one (m/z = 7733.5 Da), two (m/z = 7813.5 Da), three (m/z = 7893.4 Da) or more phosporylated residues. The conserved concentration ratios of the peptides between samples lead to high degrees of correlation of the signal intensities of the respective peptide pairs.

FIGURE 4 shows a schematic example of a correlation associated network (CAN) according to the present invention. Any CAN starts from a hub peptide and any member of a 1st order neighborhood of such a hub peptide of 1st order can be a hub peptide for the next order neighborhood and so forth.

FIGURE 5 shows a flow chart schematizing the procedural steps of an application of the CAN Module according to the present invention.

FIGURE 6 shows a graphical representation of an exemplary peptide topology of a sample, wherein peptides are represented by bullets and their mutual relations by lines connecting these bullets. Such a peptide network can be projected onto a peptide map like Figure 2 for a more intuitive analysis of the results.

FIGURE 7 shows a flow chart schematizing the procedural steps of an interaction of the Sequence Network Module with the CAN Module according to the present invention.

FIGURE 8a shows a flow chart schematizing the process of checking whether a predicted sequence matches the experimental properties of an unknown peptide.

FIGURE 8b shows a flow chart exemplifying the generation of sequence predictions, which are checked according to Figure 8a.

FIGURE 8c shows a flow chart schematizing the query of all unknown peptides P2 which are related to a known peptide P1. Sequence predictions are generated for any unknown peptide P2 according to Figure 8b.

FIGURE 8d shows a flow chart exemplifying the iteration of the process as demonstrated in Figure 8c for any peptide P1 with a known sequence.

FIGURE 9 shows a table of the monoisotopic and the average mass changes of a peptide upon the respective modification.

FIGURE 10 shows a table with exemplified motifs of chemical and enzymatic reactions, their respective mechanism/enzyme and the resulting average mass difference of the modified peptide.

FIGURE 11 shows a table listing the most common amino acids, their three- and one letter codes as well as their monoisotopic and average mass in their dehydrated form.

FIGURE 12 shows a table listing the common amino-terminal and carboxy-terminal groups of peptides, as well as the chemical composition, their respective monoisotopic and average mass.

FIGURE 13 shows a table that refers to the fraction shifts of peptides that are caused by addition of the respective amino acid to the peptide sequence under the described experimental settings with cerebrospinal fluid as sample source.

FIGURE 14a shows a table with amino acids and their empirically derived occurrence before the N-terminal cleavage position (start position) of a peptide in a precursor sequence, the respective overall occurrence of the given amino acid in all determined sequences and the ratio thereof.

FIGURE 14b shows a table with amino acids and their empirically derived occurrence after the N-terminal cleavage position (start position) of a peptide in a precursor sequence, the respective overall occurrence of the given amino acid in all determined sequences and the ratio thereof.

FIGURE 14c shows a table with amino acids and their empirically derived occurrence before the C-terminal cleavage position (end position) of a peptide in a precursor sequence, the respective overall occurrence of the given amino acid in all determined sequences and the ratio thereof.

FIGURE 14d shows a table with amino acids and their empirically derived occurrence after the C-terminal cleavage position (end position) of a peptide in a precursor sequence, the respective overall occurrence of the given amino acid in all determined sequences and the ratio thereof.

FIGURE 15 shows a flow chart schematizing the procedural steps of an interaction of the Differential Network Module with the CAN Module according to the present invention.

FIGURE 16 shows a flow chart schematizing the procedural steps of an interaction of the Marker Panel Network Module with the CAN Module according to the present invention.

FIGURE 17 shows a flow chart schematizing the procedural steps of an interaction of the Surrogate Network Module with the CAN Module according to the present invention.

FIGURES 18a and 18b show a table of the signal intensity values of the peptides with coordinates Fraction 54; m/z 2743.0, Fraction 54; m/z 1371.5, Fraction 56; m/z 2927.2 and Fraction 20; m/z 1114.3 taken from 74 samples. Furthermore, the number of related peptides k with a Spearman's Rank Order Correlation Coefficient threshold of $|r| \geq 0.8$ is shown.

FIGURE 19 shows a table with the measures of correlation of the signal intensities of the peptide with coordinates Fraction 54; m/z 2743.0 with some exemplary peptides using different measures of correlation.

FIGURE 20 shows a histogram of Spearman's Rank Order correlation coefficient probabilities. The value of a correlation coefficient of a peptide-to-peptide relation (x-axis) is plotted versus the probability for that peptide-pair to achieve that value (y-axis). Peptide-to-peptide pairs with low absolute correlation coefficients are most likely not related. This is expressed by the maximum at zero of peptide-to-peptide relations from random data (P(r) Simulation). True positive relations are very likely to be found at higher absolute correlation coefficients. Therefore the plot of correlation coefficients of peptide-to-peptide relations from measured data (P(r) Measurement) deviates from P(r) Simulation, because correlation coefficients of functionally related peptides are most likely higher than those obtained from random data. Such a plot should be generated when a threshold for a given CAN has to be chosen in order to exclude as much false positive peptide-to-peptide relations while including as many true ones as possible.

FIGURE 21 shows a table of identified peptides related to Chromogranin A 97-131, the Spearman's Rank Order Correlation coefficient value of the said peptide with the related peptide, their relative monoisotopic mass and their amino acid sequence.

FIGURE 22 shows a graph exemplifying the usability of a Differential Network of the peptides SG I 88-132 and Chromogranin A 97-131. In hypothetic patients before prostatectomy (black triangles) a correlation between these peptides is present ($r = 0.97$), and a signal intensity ratio of about 10/1 is conserved. In hypothetic samples after prostatectomy (white squares) this ratio is not present and the Secretogranin I/Chromogranin A relation is "broken".

FIGURES 23a and 23b show a table of the signal intensity values of the peptides with coordinates Fraction 54; m/z 1371.5, Fraction 56; m/z 2927.2 and Fraction 20; m/z 1114.3 of 74 samples after removal of the variance of the signal intensity of the peptide with coordinates Fraction 54; m/z 2743.0. Furthermore, the number of related peptides k with a Spearman's Rank Order Correlation Coefficient threshold of $|r| \geq 0.8$ after the removal of said variance is shown.

FIGURE 24a shows a graph plotting the signal intensity of the peptide in fraction 54 with a mass-to-charge-ratio of 2743.0 (F 54; m/z 2743.0) versus the signal intensity of the peptide in fraction 20 with a mass-to-charge-ratio of 1114.3 (F 20; m/z 1114.3). This plot exemplifies a pair of peptides showing no correlation.

FIGURE 24b shows a graph plotting the signal intensity of the peptide in fraction 54 with a mass-to-charge-ratio of 2743.0 (F 54; m/z 2743.0) versus the signal intensity of the peptide in the same fraction with a mass-to-charge-ratio of 1371.5 (F 54; m/z 1371.5). This plot exemplifies a correlation between a peptide-to-peptide pair consisting of a single charged and a double charged peptide ion.

FIGURE 24c shows a graph plotting the signal intensity of the peptide in fraction 54 with a mass-to-charge-ratio of 2743.0 (F 54; m/z 2743.0) versus the signal intensity of the peptide in fraction 56 with a mass-to-charge-ratio of 2927.2 (F 56; m/z 2927.2). This plot exemplifies a peptide-to-peptide pair exhibiting a functional relation.

FIGURE 25a shows a graph plotting the studentized signal intensity of the peptide in fraction 54 with a mass-to-charge-ratio of 2743.0 (F 54; m/z 2743.0) versus the studentized signal intensity of the peptide in fraction 20 with a mass-to-charge-ratio of 1114.3 (F 20; m/z 1114.3), i.e. the peptide pair of Figure 24a. A minimum spanning tree algorithm was performed to connect the nearest vertices. The path with the most vertices, i.e. the MST diameter, has been higlighted by a bold line. In this example, the path comprises 29 vertices.

FIGURE 25b shows a graph plotting the studentized signal intensity of the peptide in fraction 54 with a mass-to-charge-ratio of 2743.0 (F 54; m/z 2743.0) versus the studentized signal intensity of the peptide in the same fraction with a mass-to-charge-ratio of 1371.5 (F 54; m/z 1371.5), i.e. the peptide pair of Figure 24b. This plot exemplifies a correlation between a peptide-to-peptide pair consisting of a single charged and a double charged peptide ion. A minimum spanning tree algorithm was performed to connect the nearest vertices. The path with the most vertices, i.e. the MST diameter, has been highlighted by a bold line. In this example, the path comprises 50 vertices.

FIGURE 25c shows a graph plotting the studentized signal intensity of the peptide in fraction 54 with a mass-to-charge-ratio of 2743.0 (F 54; m/z 2743.0) versus the studentized signal intensity of the peptide in fraction 56 with a mass-to-charge-ratio of 2927.2 (F 56; m/z 2927.2), i.e. the peptide pair of Figure 24c. This plot exemplifies a peptide-to-peptide pair exhibiting a functional relation. A minimum spanning tree algorithm was performed to connect the nearest vertices. The path with the most vertices, i.e. the MST diameter, has been highlighted by a bold line. In this example, the path comprises 40 vertices.

FIGURE 26 shows a table where the peptides with coordinates Fraction 54; m/z 1371.5 and Fraction 56; m/z 2927.2 were tested according to a method of the present invention, whether the given peptides are potentially n times charged ions of the peptide with coordinates Fraction 54; m/z 2743.0.

FIGURE 27 shows the precursor sequence of the "Hypothetical Precursor" (HP) using a one-letter code. The sequence of the peptide HP 25-48 is underlined, the sequence of HP 25-50 is in bold letters.

Detailed Description of the Invention

Prior to giving a detailed albeit exemplary description of embodiments of the present invention the following definitions are provided to establish how the technical terms are to be understood herein.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. As used herein, the following terms have the meaning ascribed to them unless specified otherwise.

"Sample" refers to any material, substance or the like containing or potentially containing peptides.

"Peptides" refers to polymers of amino acids coupled by peptide bonds comprising at least two amino acids. These amino acids can be the twenty standard amino acids and additionally unusual amino acids as known in the arts including D- and L- amino acids. Peptides can contain additional modifications such as posttranslational, enzymatic and/or chemical modifications.

"Status of a sample or an organism" means that the status or type of a sample at the time of the generation of the sample, e.g. the drawing of blood, is reflected by the contents and the activities of the sample. The actual status of an organism at the time of sample generation (such as the drawing of blood) is reflected in the contents and activities present in the sample. The sample conserves the status similar to a snap shot picture. A status for example can represent the presence or absence of a certain disease, the presence or absence of pregnancy, the sex of the individual from which the sample originated, the presence of a certain genetic variation such as the knockout of a gene or a polymorphism, the over expression or increased activity of a certain gene or gene product (for example as a consequence of a drug or of the transfection of the gene coding for the gene product or by direct addition of the gene product, etc.), the suppression of the expression or activity of a certain gene or gene product (for example as a consequence of a drug, anti-sense nucleotides, RNAi (RNA interface) nucleotides, ribozymes, triplex-forming nucleotides, antibodies, etc.), the presence of genetic modified ingredients in food, cosmetics or other products, the age of the organism from which the sample originated, the species of the organism from which the sample originated, a certain treatment of the organism from which the sample originated (for example with a therapeutically active substance a food ingredient or substance present in cosmetics, treatment with insecticides, pesticides or other toxic substances, etc.), the geographic origin of the sample, the development stage of the organism from which the sample originated (for example the stage of a fertilized egg, an embryo, an adult, intracellular/extracellular bacteria/virus, egg/larva/pupal/adult-stage of for example butterflies, different development stages of plasmodium, etc.), the metabolic state of the organism from which the sample originated (for example hibernation, stages of the circadian rhythm, etc.), the point of time before, during or after the treatment of an organism with a substance, the localization (or tissue) within the organism, from where the sample was taken, and the like.

"Measurement parameter of a peptide" refers to any parameter known to or measurable by the investigator such as the molecular mass of the peptide, the mass/charge ratio of the peptide, the signal intensity of the measured peptide, the actual concentration of the measured peptide, the fraction-number in which the peptide is present as a consequence of a certain separation protocol subjected to the sample, or the measured activity of the peptide.

"Correlation" or "relation" refers to a hypothesized mutual dependency of at least one parameter of two peptides, may this dependency be symmetric or asymmetric, known or not known, statistically significant or not. Relations of two peptides can be caused by chemical and biochemical reactions from one peptide to the other, by concerted gene regulation of the analytes, by common precursor peptides and so on.

"Measure of correlation", "correlation measure" or "measure of association" refers to statistical means to describe the symmetric or asymmetric statistical dependency of measurement parameters of pairs of peptides in terms of their "relation". Examples for measures of correlation are: "Pearson Product-Moment Correlation Coefficient", "Spearman's Rank-Order Correlation Coefficient", "Kendall's Tau", "Kendall's Coefficient of Concordance", "Goodman and Kruskal's Gamma", "Manhattan distance", "Euclidean distance" and "Minimal Spanning Tree Diameter".

"Correlation associated network (CAN)" refers to the complete network of all measures of correlation identified within samples representing one status or identified within different groups of samples representing different statuses. It is possible that more than two peptides correlate to each other and a CAN contains at least two peptides correlating to each other. It should be noted that the peptide CAN based on "a sample" does not necessarily comprise results obtained from a single experiment. Rather, to completely determine a peptide CAN, multiple experiments are often needed, and the combined results of which are used to construct the peptide CAN for that particular sample. The results of the calculation of a CAN (CAN of first order) can be used for another round of calculation of measures of correlation and so on. The results of these kind of calculations are also termed CANs or more specifically CANs of second or higher order.

"Peptide-topology" refers to the entirety of measured and computed peptide data of a sample ("measurement parameter of peptides") comprising the masses of the peptides, the signal intensity of the peptides (preferably measured by mass spectrometry or another measurement method suitable to quantify peptides), the fraction number (if the sample was fractionated prior to mass spectrometry) and measures of correlation calculated using these data.

"Groups of samples" refers to a set of samples corresponding to a certain status. A group of samples for example could comprise 10 plasma samples of diabetic patients. The samples of a group need not to be of exactly the same origin. For example a group of samples may also comprise 5 plasma samples of diabetic patients and 5 urine samples of diabetic patients. The reason for this being that many peptides present in plasma are also present in urine and for example the same diabetes-specific peptides may be present in plasma and urine, as long as the sample originates from a diabetic patient.

"Known peptide" means that the peptide with that particular sequence or part of a sequence in the sample is known to the user of the invention. An unknown peptide is a peptide whose sequence is not known to the user of the invention, although the sequence of the peptide may be known from the literature or other sources of information such as sequence data bases.

"Potential peptide" refers to a mass spectrometric signal which most likely represents a peptide.

"Precursor of a peptide" refers to the longest amino acid sequence present in nature comprising the sequence of the peptide, i.e. form which the peptide can originate.

"Coordinate(s) of a peptide" refer to the mass-to-charge ratio and optionally further specific measurable properties obtainable by a detection or identification process that are involved in the identification and/or quantification of the said peptide/peptide ion. In the examples of this invention the peptide coordinates are the elution time/fraction number of a chromatographic process and the mass-to-charge ratio, thus comprising of two coordinates. In this invention, these coordinates often are written in a short form, such as "F 56; m/z 2873.0", which identifies the signal of a peptide found in fraction 56 with the mass-to-charge ratio 2873.0. Of course, further dimensions can be necessary, such as a previous capillary electrophoresis, or a downstream second mass spectrometric process. "Coordinates of a peptide", "signal coordinates" or "peptide" are often used synonymously.

"Fitness value" refers to an assessment of a predicted sequence based on the experimental properties of an unknown peptide. Any predicted sequence gains points for properties that match the experimental properties, such as the correct prediction of the fraction number. The higher the "fitness value", the more probable the correctness of the predicted sequence. According to the present invention fitness values are manually or automatically suggested for each sample type and empirically tested for suitability.

"Landmark peptides" refers to peptides that are related to numerous other peptide signals and least related to each other. The identification, e.g. sequencing, of these landmark peptides should be prioritized to gain a rapid overview about the peptide composition of a sample.

Providing the data

Figure 1 shows schematically the hardware components and software modules according to the present invention, their interfaces and the flow of information between the hardware components and the software modules. Although measurement data could be provided without fractionating the samples prior to performing mass spectrometry, such a fractionation e.g. by chromatography into e.g. 96 fractions is preferred. A "fraction" in terms of chromatography is a part or the effluent recovered during a separation step. Usually, several fractions are collected. Fractions usually contain different "subsets" of peptides from the sample. Suitable separations methods for peptides are chromatographic methods such as ion exchange, hydrophobic interaction, iso-electric focusing, gel filtration or affinity chromatography, electrophoretic methods such as native, iso-electric, denaturizing or SDSgel electrophoresis using matrixes such as polyacrylamide or agarose gels, paper electrophoresis, thin layer chromatography, capillary electrophoresis, methods using centrifugation for separation such as sucrose or Caesiumchloride gradient centrifugation and the like. These chromatographic fractions are then subjected to a measurement of the mass spectrum providing 96 mass spectra, which can be visualized e.g. in a 2D gel-like format as shown in Figure 2. To this end all kinds of methods suitable to determine masses of peptides and preferably all kinds of mass spectrometry can be used in the present invention such as matrix-assisted laser desorption time of flight (MALDI-TOF) mass spectrometry, liquid chromatography electro-spray ionization (ESI) quadrupole time of flight mass spectrometry (LC-ESI qTOF) and the like. It is furthermore possible to analyze only selected but not all fractions by mass spectrometry.

Each bar in Figure 2 depicts a peak present in one of the 96 mass spectra, wherein the colour intensity of the bar corresponds to the intensity of the corresponding mass spectrometric signal. The x-axis of Figure 2 represents the mass to charge ratio m/z and the y-axis the chromatographic fraction number. The m/z values preferably range from 1.000 to 15.000, although higher or lower m/z values can be included as long as these values can be resolved by mass spectrometry or other methods. Within this range of m/z values the detected peptides can be comprised of only two amino acids at the lower end up to peptides of very large molecular mass such as alpha-2 macroglobuline having a molecular mass of 725 kDa.

Similar 2D gel-like maps are produced for every sample out of the set of samples to be analyzed. These maps can be averaged yielding an averaged peptide mass fingerprint map as shown in Figure 2. This averaged map serves as a template for the definition of usually about a thousand peak coordinates, i.e. the x-coordinate corresponding to the m/z value and the y-coordinate corresponding to the fraction number. In practice one selects those peak coordinates exhibiting a signal above a certain threshold value.

Data Pre-processing

In order to obtain measurement data that is suitable for a correlation analysis and that gives meaningful results preferably a pre-processing of the data is performed using methods such as baseline correction, spectra normalization, outlier detection and the like. Methods for baseline correction are well known in the art (e.g. Fuller et al, *Applied Spectroscopy*, 42, 217 1988). In a preferred embodiment the pre-processing of the data is performed by applying the baseline correction being part of the software RAZOR Library 4.0, Spectrum Square Associates, Ithaca NY, USA. Optionally a normalization of the mass spectra can be performed by using the signal intensities or the integrated mass spectra. Outlier samples can be identified by means of a principal component analysis as provided by the commercially available software package Pirouette 3.0, Infometrix Inc., WA, USA. Based on this principal component analysis individual mass spectra or even whole samples that exhibit a Mahalanobis distance M_D above a critical threshold value of should not be considered for a further analysis and thus be discarded. In the examples described further below a Mahalanobis distance of $M_D > 11.5$ was chosen for 74 samples.

The preprocessing, processing and display of the data according to the present invention can be performed e.g. on a Apple G4 Computer, wherein the CPU consists of 2 processors with 800 MHz each and the memory size is 1.25 Gigabyte. The local data storage of peptide-to-peptide relations (measures of correlation, coordinates of peptides) is accomplished by a local Valentina Database system (Valentina 1.9 for Realbasic, Paradigma Software, Beaverton, Oregon, USA). The peptide sequence information is provided by a proprietary Interbase Server (Interbase 6, Borland Software Corp., Scotts Valley, CA, USA). Microsoft Internet Explorer 5.1 for Apple computer systems can be used for representation of results from internet resources. The CAN software launches the Internet explorer with a specific address that contained the keywords for querying the Swiss-Prot, the PubMed, and the US Patent database. Visualization of three-dimensional objects can be performed using a Realbasic RB3D engine (RealBasic 3.5, RealSoft, Austin, Texas, USA).

Other digital computer system configurations can also be employed to perform the methods of the present invention, and to the extent that a particular system configuration is capable of performing the method of this invention, it is equivalent to the representative digital computer system schematically shown in Figure 2. Once they are programmed to perform particular functions pursuant to instructions from program software that implements the methods of the present invention, such digital computer systems in effect become special-purpose computers particular to the methods of this invention.

Computer programs implementing the methods according to the present invention will commonly be distributed to users on a distribution medium such as floppy disk or CD-ROM. From there, they will often be copied to a hard disk or a similar intermediate storage medium. When the programs are to be run, they will be loaded either from their distribution medium or their intermediate storage medium into the execution memory of the computer, configuring the computer to act in accordance with the method of this invention. All these operations are well-known to those skilled in the art of computer systems. The term "computer-readable medium" encompasses distribution media, intermediate storage media, execution memory of a computer, and any other medium or device capable of storing a computer program implementing the methods of this invention for later access by a computer.

Correlation Associated Network Module

As exemplified by the arrows in Figure 1 the raw measurement data or preferably the preprocessed measurement data is supplied to the so called Correlation Associated Network (CAN) Module 42. Of the modules 40 of the present invention the CAN Module 42 is the most fundamental one. Basically the CAN Module 42 scans the measurement data obtained for example from Liquid Chromatography-Mass Spectrometry (LC-MS) experiments 22. On the basis of this data correlations of peptides are searched for by calculating measures of correlation between for example their relative concentration as measured by mass spectrometry.

Measures of correlation can be used to represent the degree of relationship between two variables throughout many observations. These variables can be either correlated, not correlated or anti-correlated. In the context of the present invention measures of correlation are used to detect such correlated, not correlated or anti-correlated peptides in a set of samples. This can be done e.g. by calculating Spearman's rank-order correlation coefficient of the signal intensities of two peptides measured in several samples. Preferably this is done for all pairs of peptides. Once these measures of correlation have been calculated only those pairs of peptides are selected that exhibit a certain behaviour, i.e. a certain degree of correlation, a certain degree of anti-correlation or a certain degree of no correlation at all. The parameters of such selected peptide pairs, e.g. the coordinates of the two peptides of each peptide pair, the measure of correlation, etc., can be stored, displayed on a display device or further processed. Preferably the data is stored in a database, as a text file or in another computer-readable form. Alternative measures of correlation to Spearman's rank order correlation coefficient are Pearson Product-Moment Correlation Coefficient, Kendall's Tau, Kendall's Coefficient of Concordance, Goodman and Kruskal's Gamma and Minimal Spanning Tree diameters.

A Minimal Spanning Tree (MST), also known as Minimum Spanning Tree, is defined by the collection of edges that joins together all points in a connected set of data points, with the minimum possible sum of edge values (e.g. Evan, *Graph Algorithms*, Computer Science Press, 1979). An edge can be graphically displayed by a line connecting two data points. A MST can be graphically displayed by a set of points (data points) connected by a minimum of lines to each other. Examples of MSTs are shown in Figures 25a to 25c as described in more detail further below. A MST also provides a plausible "connectionist" approach to solving the "Traveling Salesman" problem (e.g. Kruskal, *Proc. American Math. Soc.*, 7, 48-50, 1956; Sun et al, *Physica A*, 199, 232-242, 1993), which identifies the minimum connected path between all data points. The MST diameter can be defined as the maximum number of edges in the paths of a graph of a MST. Usually a correlation, for example a Spearman's rank order correlation coefficient, is used to find a measure of correlation or association or dependency between variables, i.e. data points. A problem is that correlation is sensitive to linear trend, and linear trends are not always well presented for two associated variables. In the present invention, the diameter of the MST is used as an alternative measure of correlation between two variables. In order to use the diameter of the MST to analyze a given set of n statistical observations, all observations should be connected via the MST and then the MST diameter should be calculated. The larger the MST diameter is, the stronger is the association between two variables. In the context of mass spectrometry signal intensity data (in the present invention preferably MALDI mass spectrometry signal intensity data) it was found, that MST diameters $\geq$ 0.425 times n indicate a noticeable association between signal intensities of peptide coordinates. In general all kinds of mass spectrometry signal intensity data, such as MALDI or ESI mass spectrometry data, can be used according to the present invention.

As already mentioned pairs of peptides are tested for their degree of correlation by estimating e.g. Spearman's rank order correlation coefficients between their signal intensities throughout many observations. It turns out that pairs of peptides which are biologically or functionally related surprisingly often exhibit correlation coefficients that are much higher than correlation coefficients that would be expected by chance. Unrelated pairs of peptides have low absolute values of correlation coefficients. Figure 3 exemplifies the correlational behaviour of related peptides. Four traces of spectra from four different samples are focused on the signals of a human osteopontin peptide being comprised of the amino acids 249-314 of human osteopontin (m/z = 7653.6 Da) and its phosphorylated derivatives, carrying one (m/z = 7733.5 Da), two (m/z = 7813.5 Da), three (m/z = 7893.4 Da) or more phosphorylated residues. The conserved concentration ratios of the peptides between samples leads to high degrees of correlation of the signal intensities of the respective peptide pairs.

Using the results of the above described computations of measures of correlation so called correlation associated networks (CANs) can be defined. A CAN, i.e. a network of peptide relations, comprises a peptide of interest, the so called hub peptide, and all those peptides and sample parameters that correlate to a certain degree with the hub peptide. The term hub is used in a similar manner in the theory of network topology and is to characterize the resemblance of a hub peptide to the hub of a wheel, the hub peptide being at the center of the spokes representing the peptide-to-peptide relations and the correlating peptides being at the respective ends of the spokes. In practice, the composition of a CAN is highly dependent on the threshold of correlation as selected by the user. This threshold is chosen according to the goal of a user. If a user is searching for peptides that strongly relate to a peptide of interest, such as peptides stemming from the same precursor, then he will select a threshold that will cause a selection of only the upper 5 % of the strongest correlations with the peptide of interest. The threshold value to be chosen for e.g. the Spearman's rank order correlation coefficient depends for the thus selected subset on the number of samples and the peptide of interest. In case the user is interested in finding functionally related peptides, such as e.g. peptides being co-secreted from vesicles, the user will choose a threshold value, that will select e.g. the upper 10 % of the strongest correlations.

The hub peptide and the peptides related thereto and selected as described above represent a CAN of first order. Depending on the objective it can be necessary to compute CANs of higher order due to the complexity of biological networks and pathways. As explained above, CANs connect directly related peptides which exhibit a high degree of correlation. Adjusting the threshold to lower values results in including more loosely related peptides into the network as well as increasing the probability of predicting false relations. For this reason a preferred embodiment of the present invention contemplates the computation of CANs of higher orders, such as e.g. second and third order. Since the direct members of a network of interest constitute the first order neighborhood, all these members are potential starting points for the calculation of second order neighborhoods as shown in Figure 4. Although computing CANs of higher order certainly will improve the results, the computational requirements set an upper limit, because the computational effort increases with the order of the CANs. A calculation of a CAN of the nth order, where n is greater than 5, can require more than several millions of calculations. Thus, this approach preferably should be used for the analysis of rather complex samples in order to include indirectly related peptides, thus avoiding having to decrease the value of the correlation threshold and possibly including false relations.

For any kind of sample the composition of peptides varies, novel peptide coordinates emerge, others disappear and many peptide coordinates have a different peptide sequence aligned to it. This results in dealing with many unknown peptide coordinates when operating with novel sample sources (types of samples). In order to accelerate analysis of an interest list or more general, to analyze the overall peptide composition of a sample, according to the present invention it is possible using CANs to accelerate the identification of peptides in complex biological samples by defining a list of representative peptides, so called landmark peptides, for further analysis such as peptide sequencing based on CANs described further below. The method comprises the following steps, which are shown in Figure 5. At step 80 mass spectra are provided as described above, wherein the peaks of the signal intensities in the mass spectra correspond to potential peptides. Then the measures of correlation between the measured signal intensities corresponding to potential peptides are computed (step 82). Thereafter at step 84 those peptides are grouped together that exhibit a degree of correlation above an adjustable threshold. These selected peptides constitute a CAN present in the samples analyzed. Finally, one peptide out of each determined CAN is assigned to represent that respective CAN at step 86. In doing so a plurality of landmark peptides is provided being representative of the analyzed samples. These landmark peptides have the properties of being hub peptides and they are least related to other peptides within the same type of samples. Identifying a list of these landmark or prioritized peptides gives a rapid overview about the peptide composition present in complex biological samples, omitting the majority of similar peptides from for example the same precursor peptide. This is useful to obtain a general overview of the key peptides present in a sample or present in an interest list from that sample.

It is contemplated that a such generated interest list of prioritized landmark peptides will contain a set of n peptide coordinates, and for any peptide z the number of relations, which the peptide z has at a defined threshold r, $k_{z,r}$, will be determined. The peptide z with the highest value of $k_{z,r}$ will be defined as y and be rank 1 etc. on the prioritization list. Then the variance of signal intensities of that determined peptide coordinate y will be removed from the signal intensities of the related peptides x in a data matrix, for example by a combination of formulas 1, 2 and 3 shown below. Then this peptide will be removed from the data matrix. Calculations of any k and r start from the beginning to determine the representative peptide ranked number 2 in the prioritization list, and so on. Calculations end for example when the data matrix contains no more peptide coordinates, or no peptide has more than zero relations, or the number of peptide coordinates desired has been reached.

Formulae 1 to 3: Removal of variance of the representative peptide coordinate y on peptide coordinate x

$$X_{VR,p} = X_p - a_{xy} - b_{xy} Y_p \qquad (1)$$

where

$X_{VR,p}$: Signal intensity of peptide x at observation p, Variance of peptide y removed

X_p : Signal intensity of peptide x at observation p

Y_p : Signal intensity of peptide y at observation p

m : number of observations

$$a_{xy} = \frac{\left(\sum_{p=1}^{m} X_p\right)\left(\sum_{p=1}^{m} Y_p^2\right) - \left(\sum_{p=1}^{m} Y_p\right)\left(\sum_{p=1}^{m} X_p Y_p\right)}{m\sum_{p=1}^{m} Y_p^2 - \left(\sum_{p=1}^{m} Y_p\right)^2} \qquad (2)$$

and

$$b_{xy} = \frac{\sum_{p=1}^{m} X_p Y_p - \frac{1}{m}\left(\sum_{p=1}^{m} X_p\right)\left(\sum_{p=1}^{m} Y_p\right)}{\sum_{p=1}^{m} Y_p^2 - \frac{1}{m}\left(\sum_{p=1}^{m} Y_p\right)^2} \qquad (3)$$

It is further contemplated that peptides being part of a CAN preferably are represented by graphical objects such as e.g. bullets and their mutual relations by lines connecting these bullets. In order to enable a more intuitive analysis of the results, this network can be projected onto a peptide map as shown in Figure 6. Peptides that have been identified can be provided with links to databases, with lists containing additional information about these peptides or with other sources of additional information regarding said peptides.

The coordinates or measurement parameters of related peptides can be queried in public, commercial and/or proprietary databases in order to identify further data about the potential identity, function or use of the corresponding peptides. Suitable public databases include e.g. the PubMed literature database, the OMIM disease database, the NCBI-Sequence database (all provided by the US National Library of Medicine, MD, USA), the Swiss-Prot and TrEMBL Sequence database, enzyme database, Swiss 3D image database, Prosite protein family and domain database (all provided the Swiss Institute of Bioinformatics, Switzerland), patent databases of the US, European, Japanese, German patent offices, the Gene Cards database of the Weizmann Institute, etc. Suitable commercial databases are for instance commercial patent databases containing patented amino acid or nucleic acid sequences such as DGENE (Thomson Derwent, USA) or REGISTRY (Chemical Abstracts Service, USA). A suitable proprietary database is the database of the user containing peptide sequences from various sources and species. This combination of the visualization of peptide networks and the connection to many sources of information alleviates the evaluation of the identified peptides for potential uses such as their use as therapeutic peptides or as biomarkers as will be described in more detail further below.

As is apparent from the above, correlation associated networks can be used to generate hypotheses about relations between structurally and/or biologically related peptides. These hypotheses are based on a correlational analysis of signal intensities and corresponding relative peptide concentrations from independent samples. The examples described in the sections further below will demonstrate that correlation associated networks are powerful tools for the systematic analysis and interpretation of large peptidomic and proteomic data in order to reveal functional relationships governing protein synthesis, posttranslational modifications and degradation. CANs support the discovery of novel bioactive and diagnostic peptides leading beyond the mere comparison of peptide concentration changes caused by a disease.

According to the present invention the CAN Module 42 is interacting with several application modules 44 comprising a Sequence Network Module 46, a Differential Network Module 48, a Marker Panel Network Module 50 and a Surrogate Network Module 52 as shown in Figure 1. These application modules 44 of the present invention and their interaction with the fundamental CAN Module 42 will be described in detail in the sections below.

Sequence Network Module

The interaction of the Sequence Network Module with the fundamental CAN Module according to the present invention allows to predict the amino acid sequences of unknown peptides with or without modifications of the sequence and/or to predict unknown modifications of a known or unknown peptide sequence. Although the identity of the peptide is unknown, certain physicochemical and biochemical properties of the signal of an unknown peptide are known and can be exploited for amino acid sequence prediction such as the mass-to-charge ratio (m/z) or the chromatographic behaviour (fraction number/retention time). Furthermore bioinformatic support data shown at 56 in Figure 1 such as the correlation associated network of related peptides, mass differences and differences in fraction number between the peptides of the correlation associated network, and the like are accessible as they can be computed using experimental data and the amino acid sequences of other members of the correlation associated network possibly already known.

Figure 7 shows a flow chart schematizing the procedural steps of an interaction of the Sequence Network Module with the CAN Module according to the present invention allowing the prediction of the sequence of peptides using the peptide topology of a plurality of samples containing a peptide having a known precursor. At step 80 a respective mass spectrum for each sample of said plurality of samples is provided, wherein the signal intensity peaks correspond to potential peptides. Thereafter at step 88 the peptide having a known precursor is identified using the mass of said peptide, wherein the sequence of the known precursor is known. Then measures of correlation between the signal intensity of the peptide having a known precursor and the signal intensities of the other potential peptides are computed at step 90. At step 92 those potential peptides are selected, which exhibit a degree of correlation with the peptide having a known precursor above a certain adjustable threshold, and finally the sequence of the potential peptides are predicted at step 94 by matching masses of putative fragments of the sequence of the known precursor with the masses of the potential peptides correlating with said peptide having a known precursor.

Alternatively after step 92 the mass differences between each of the potential peptides and the known peptide can be computed at step 96, and thereafter the sequence and/or the biologically, chemically or physically modified sequence of the potential peptides predicted at step 98 by using data about mass differences caused by biological, chemical or physical processes matching the mass differences determined in step 96.

The first of the above approaches is more comprehensive, since all plausible putative sequences are generated from the precursor sequence of the known peptide (steps 90-98). The second approach (steps 90-96, 100-102) generates fewer but more reliable predictions. It has been observed that related peptides very often have very similar sequences/sequence modifications, and these predictions are promoted by the second approach. Nevertheless, since both approaches have steps 90-96 in common, computational power is "saved" if both approaches are combined in one operation, as contemplated in the present invention.

Mass differences may result from addition or removal of N- or C-terminal amino acid residues or of postranslational modifications of amino acid side chains such as phosphorylation, amidation, sulfatation, glycosylation, fatty acids or Ubiquitin modification, and the like or chemical modifications such as oxidation, disulfide bonding, and the like or N- or C-terminal modifications such as pyroglutamate modifications and the like. All of these modifications result in distinct increases or decreases of the molecular mass of the corresponding peptide. Also internal insertions or deletions or the exchange of one amino acid for another, so called point mutations, result in exactly predictable mass changes of the peptide.

According to the present invention the prediction of sequences is possible regardless of whether the identity of one of the related peptides is known or not. Especially if the identity of one peptide is known, mass differences corresponding to the molecular masses of amino acid residues allow to directly predict the complete sequence of the unknown peptide with high reliability. If the identity of no peptide is known, than for example it can be predicted that the unknown peptide 1 and the unknown peptide 2 are identical, except that for example peptide 2 contains an additional amino acid residue, for example a Tyrosine residue, or for example peptide 2 is the same peptide as peptide 1 except that it is phosphorylated, etc. The prediction is not always correct, but the more independent information is accessible, the more reliable the prediction becomes. For example if the mass difference fits to the addition of an Tyrosine amino acid residue and the peptide is present in a fraction, which fits to the prediction of the fraction-shift of a peptide with an additional Tyrosine residue, the overall reliability of the prediction increases.

For this embodiment the use of proprietary and/or commercial and/or public databases is possible. Suitable databases are for example databases containing amino acid or nucleic acid sequence information such as the NCBI sequence data base, Swiss-Prot, the EMBEL sequence data base, the DNA data base of Japan, data bases of patented sequences, and the like, data bases containing information about the structure of carbohydrates, such as PROSITE (Falquet et al, *Nucleic Acids Res.*, 30, 235-238, 2002), data bases containing information about postranslational, enzymatic or chemical peptide modifications such as phosphorylation sites of peptides, glycosylation sites of peptides, positions of unusual amino acids such as hydroxy-proline or hydroxy-lysine within peptides, databases containing information about recognitions sites of proteases, ligases, phophatases, kinases, and the like within peptide sequences, databases containing information about the susceptibility of certain amino acids or sequences of amino acids towards chemical modifications such as oxidation, reduction, intra-molecular rearrangement, data bases containing data about three-dimensional structures about peptides, carbohydrates or other biological structures, and the like (Falquet et al, *Nucleic Acids Res.*, 30, 235-238, 2002). All of these different kinds of databases enable to predict the structural difference between peptides, based on certain incremental increased or decreased molecular masses of these peptides. For example:

(i) amino acid sequences stored in databases allow the calculation of the masses of successive shortened or extended peptides or of peptides containing mutations of their sequence

(ii) databases containing for example recognition sites (sequences) of kinases allow to predict, that the molecular mass of a certain peptide, containing such a recognition site, may have a molecular weight increased or decreased by the mass of a phosphate group

(iii) data bases of recognition sites of proteases allow to predict the molecular masses of potential proteolytic fragments of a certain peptide

(iv) databases containing experimental data about physical properties of peptides such as elution times during for example hydrophobic interaction chromatography allow to predict, if a certain peptide sequence with a certain molecular mass is likely to elute at a certain time point during chromatography

(v) databases containing prediction values of chromatographic retention times or fraction numbers based on the amino acid composition and/or sequence of the peptide: if a certain chromatographic column is used, a peptide with an additional tyrosine residue would elute 3 fractions later than a peptide without that additional tyrosine residue. For example a peptide I in fraction x with mass y is known and a related peptide II within fraction x+3 has the molecular mass y plus the mass of a tyrosine residue. This would indicate with high reliability that peptide II is the same peptide as peptide I, except that it contains an additional tyrosine residue somewhere within its sequence

(vi) databases of three-dimensional structures of for example peptides allow to predict, if there is for example space enough at a certain amino acid side chain to be modified for example by a phosphate group or a sugar moiety, resulting in an increased molecular weight of the potential corresponding peptide

The prediction of physicochemical and biochemical properties of putative amino acid sequences fit surprisingly well to experimentally determined properties. This approach can be extended by utilizing knowledge about precursor amino acid sequences and posttranslational, chemical and enzymatic modifications of known related peptides as provided by the support data 56 shown in Figure 1 and as discussed above. Furthermore, information about a known peptide such as the name of its precursor, its precursor sequence, its start and end-position within the precursor sequence can be retrieved before or during the prediction processes. Information about protease recognition sites, predictions of domains, and structures sensitive to proteolytic digestions can also be retrieved. This information can be supplied manually, from databases or lists or from a comparable source of information. A conversion of mono-isotopic m/z ratio to average m/z ratio, from the m/z ratio of the charged ion to the m/z ratio of the un-charged ion within a reasonable error tolerance is known to those skilled in the art.

The invention comprises specific rules, which determine if a putative amino acid sequence derived according to one of the methods described above fits to the peptide signal coordinates of an unknown peptide. These rules which are schematically shown in Figures 8a to 8d can be applied in any order and it is not necessary to apply all of them in any given case:

Rule a:

This rule applies formula 4 (shown below) to check, whether the unknown peptide coordinate is an n-fold charged ion of the known peptide coordinate by the following condition, where n can be an integer number greater than 1, $m/z_{\text{unknownpeptide}}$ is the m/z ratio of the unknown peptide, $m/z_{\text{knownpeptide}}$ is the m/z ratio of the known peptide and $\text{Mass}_{\text{Threshold}}$ is a maximum difference of the calculated mass from the measured mass. A preferable $\text{Mass}_{\text{Threshold}}$ equals the mass precision of the instrument and the subsequent data processing routines. If this condition is met, the proposal is rewarded with a high fitness value and the proposal that the unknown peptide is the n-fold charged ion of the known peptide can be stored.

Formula 4: Check for n times charged peptide ions

$$\left| n*(m/z_{unknownpeptide} - 1) - m/z_{knownpeptide} \right| = Mass_{deviation} \leq Mass_{threshold}$$

Rule b:

If the difference of the masses of a known hub peptide P1 and a related peptide P2 corresponds to a mass of an post-translational modification, as listed for example in the table "Mass Changes Due to Post-translational Modifications of Peptides and Proteins" shown in Figure 9 or as known from the prior art (Falquet et al, *Nucleic Acids Res.*, 30, 235-238, 2002), then P2 is proposed to be the post-translationally modified derivative of P1. If the amino acid sequence of the known hub peptide P1 contains specific sites for posttranslational modifications or it is known that P1 is or can be posttranslationally modified, and if the mass difference between the known and the unknown peptide corresponds to the mass difference resulting from the presence or absence of that posttranslational modification, the fitness value is increased. The table shown in Figure 15 exemplifies motifs, enzymes recognizing these motifs and the resulting mass differences. Numerous other postranslational modifications or putative sequence motifs bearing certain post-translational modifications are known in the prior art and could be used as well such as N-glycosylation or O-glycosylation sites (motifs), phosphorylation sites, sulfatation sites, and the like (e.g. Alberts et al, *Molecular Biology of the Cell,* Garland Publications, 2002; Coligan et al, *Short Protocols in Protein Science*, John Wiley & Sons, 2003; Falquet et al, *Nucleic Acids Res.,* 30, 235-238, 2002).

Rule c:

Putative sequences or putative fragments are generated from potential amino- and carboxy-terminal truncations or additions of amino acids of the known precursor sequence of the hub peptide and are checked whether they match the found m/z ratio of the unknown peptide coordinate. A putative sequence is generated by systematically and iteratively defining start- and end-positions, i and j, in the given precursor sequence of the hub peptide, as exemplified in Figure 8b. The mass of the putative amino acid sequence M_{CALC} is calculated by summing up the masses of the amino acids, the hypothesized postranslational modifications of the amino acid residues and/or of the terminal groups of the putative amino acid sequence (see the tables in Figures 9, 11 and 11 and formula 5 shown below). Rule c defines that if the calculated mass differs from the measured mass M_{FOUND} of the unknown peptide signal by less than a given mass threshold T_{Mass}, this putative amino acid sequence plus posttranslational modifications are proposed and further rules d to i can be applied, otherwise this proposal is rejected. This can be done with one or more putative peptide sequences or with all hypothetically possible peptide sequences that can be deduced from the precursor sequence of the known related peptide signal coordinates.

Formula 5: Calculation of Masses

$$M_{CALC} = n_A * M_A + n_R * M_R + n_N * M_N + n_D * M_D + n_C * M_C + n_E * M_E + n_Q * M_Q + n_G * M_G + n_H * M_H + n_I * M_I + n_L * M_L + n_K * M_K + n_M * M_M + n_F * M_F + n_P * M_P + n_S * M_S + n_T * M_T + n_W * M_W + n_Y * M_Y + n_V * M_V + M_{N\text{-}Terminal\ Group} + M_{C\text{-}Terminal\ Group} + M_{Modifications}$$

wherein:

M_{CALC} is the calculated mass of the peptide with the given/putative sequence,

$M_{ONE\ LETTER\ AMINO\ ACID\ CODE}$ is the mass of the appropriate amino acid,

$n_{ONE\ LETTER\ AMINO\ ACID\ CODE}$ is the number of the appropriate amino acid in the given/putative sequence,

$M_{N\text{-}Terminal\ Group}$ is the mass of the N-terminal group,

$M_{C\text{-}Terminal\ Group}$ is the mass of the C-terminal group, and

$M_{Modifications}$ is the mass change by modification(s), in the case of no modification $M_{Modifications} = 0$.

Rule d:

The number and the identity of amino acids influence the elution time/fraction number, depending on the size and the kind of the chromatography column used and the chromatography conditions. The fraction number/elution time of a peptide can be surprisingly well predicted on the basis of its amino acid sequence by the so called Group Method of Data (GMDH, e.g. Mueller and Lemke, *Self-Organising Data Mining Extracting Knowledge From Data*, Trafford Publishing, 2003), multiple regression or comparable mathematic methods with a training set of peptides with known sequences, which are separated under the same chromatographic conditions as exemplified in Formula 6 shown below. In the said training set, the number of any amino acid residue type of a peptide is the independent variable whereas the fraction number of the peptide is the dependent variable. If the calculated fraction number (e.g. Formula 6) of the predicted amino acid sequence matches the derived fraction number of the unknown peptide within a given error tolerance, then the model fitness points are increased. If the mass differences are proposed to be resulting from distinct amino acid deletions/additions and if the differences in fraction number can be matched with these said amino acid sequence differences (see Figure 13), the model fitness points are increased.

Formula 6: Estimation of fraction number based on proposed sequence

$$F_{CALC} = 35.89 - 0.45 * n_S + 0.47 * n_E + 2.86 * n_I - 3.82 * n_H + 5.15 * n_L + 5.54 * n_F + 2.92 * n_Y - 1.72 * n_K - 0.85 * n_Q + 5.35 * n_W + 2.20 * n_V$$

wherein:

F_{CALC} is the calculated Fraction number of the given sequence, and

$n_{\text{ONE LETTER AMINO ACID CODE}}$ is the number of the appropriate amino acid in the given sequence.

Rule e:

If the N-terminal position of the predicted amino acid sequence is the same as the N-terminal position of the known peptide, the fitness value is increased. This is because the known peptide and the unknown peptide of the underlying signals are related via a C-terminal proteolytic reaction, which is observed surprisingly often.

Rule f:

If the C-terminal position of the predicted amino acid sequence is the same as the C-terminal position of the known peptide signal, the fitness value is increased. This is because the known peptide and the unknown peptide of the underlying signals are related via an N-terminal proteolytic reaction, which is observed surprisingly often.

Rule g:

If the start position and/or the end-position of the predicted sequence is preceded or followed by sites of infrequent proteolytic events, the fitness value of this proposal is decreased. If the start position and/or the end-position of the predicted sequence is preceded or followed by sites of frequent proteolytic events, the fitness value of this proposal is increased. This is because it has been observed that peptides are often products of specific and/or unspecific proteases. Depending on the source and preparation procedure of the samples, proteases and intra-molecular rearrangements, such as disulfide bonding, can vary. With for example liquor cerebrospinalis (CSF) as sample source, the sequences "R-R" or "R-K" are frequently preceding a peptide's N-terminal position in a precursor as they are recognition sites of the prohormone convertase PC2 in CSF. Next to known enzyme recognition sites, some amino acids are more frequently and others are less frequent. Positions preceding or following N- and C-terminal positions of peptides can be predicted on the basis of their mere percentage occurrence in a particular sample treated in that particular way. This kind of information can easily be determined empirically and an example for peptides present in human liquor cerebrospinalis is shown in the tables in Figures 14a to 14d. The tables "CSF: amino acid Before First Cleavage", "CSF: amino acid After First Cleavage", "CSF: amino acid Before Last Cleavage", and "CSF: amino acid After Last Cleavage" summarize empirically found N- or C-terminal amino acid frequencies as a result of proteolytic processes. Rule h increases the fitness value when those amino acids at the top of the tables shown in Figures 14a to 14d are present at the corresponding positions in the predicted sequence, while those amino acids at the bottom of these tables decrease the fitness value of the prediction. The tables shown in Figures 14a to 14d are suitable to predict the likelihood of the presence of certain amino acid residues at the N- or C-terminus of peptides present in human liquor cerebrospinalis as long as the CSF samples are treated in the same way as the CSF samples of the examples of the present invention. Tables similar to the ones shown in Figures 14a to 14d can be generated empirically for any sample such as whole blood, serum, plasma, urine and the like, and the treatment of the samples can be of any kind, as long as all samples are treated in the same way.

Rule h:

If the mass difference between the peptide coordinates of a known and an unknown peptide can be explained by the loss of one or more distinct N- or C-terminal amino acids, the fitness value of this prediction is increased.

Rule i:

If a prediction has been generated by one of the rules b to h or a combination thereof, proposing that the unknown peptide is a reactant or a product of a post-translational modification of the known peptide, this proposal is tested by determining in terms of accessibility of the reaction site within the protein sequence by an enzyme performing the given post-translational modification. Thus, if a look-up in a database storing three-dimensional data of peptides or proteins reveals that the proposed site is on the surface of the protein and/or its conformation sterically allows action of that enzyme, the fitness value of that prediction is increased. In the same way, if a region of a sequence is proposed to be modified by a post-translational modification process, the accessibility of that sequence region to enzymes is assessed by means of algorithms estimating the hydrophobicity of that particular region (Engelman et al, *Ann. Rev. Biophys. Chem.*, 15, 321, 1986; von Heijne, *Eur. J. Biochem.*, 116, 419, 1981). For example, a highly hydrophilic sequence region is more likely to be accessible by enzymes performing post-translational modifications than a hydrophobic sequence region, thus the fitness value of that prediction is increased.

The results computed by applying rules a to i and optionally additional rules can be stored in a list or a database in computer readable format and/or can be printed or displayed via an appropriate user interface such as a monitor. If more than one prediction for an unknown amino acid sequence fits the results obtained with the rules described above, then the predicted sequence can be ranked with the best fitting sequence for the unknown peptide on top as shown at step 148 in Figure 8b. If the known peptide P1 has more than one related, unknown peptide P2, than the approach described can be repeated for all unknown peptides P2 as shown in Figure 8c. The approach described above can be extended to any known peptide signal P1 in a list of peptides as exemplified in Figure 8d.

Differential Network Module

According to the present invention the interaction of the Differential Network Module with the fundamental CAN Module allows to identify peptides which independently from each other distinguish between a sample A and a sample B. A status can be young, old, healthy, diseased, sweet taste, bitter taste, transfected, non-transfected, yellow, green, male female, pregnant, non pregnant, smoker, non smoker or any other criterion defining a group or a subgroup of samples or organisms from which samples are derived. Optionally the Differential Network Module is linked with various databases, containing data such as the status of the samples, as well as with the other modules of the present invention and especially the basic CAN Module as shown in Figure 1. The Differential Network Module instructs the CAN Module to define subgroups of samples defined by distinct criteria, such as the status of the samples, and further to calculate separately the peptide-to-peptide relations for any status or any combination of more than one status. First, those peptide pairs that suffice a threshold of correlation in a group of samples representing the status A, second, those peptide pairs that suffice a threshold of correlation in a group of samples representing the status B, and third, relations can be defined on the basis of differences between the correlations of the compared status A and status B. If a user is interested in peptide-to-peptide relations, that are most different in samples from two different statuses A and B, then he will search for peptides where the correlation coefficients of the respective peptide-to-peptide relations is different, and where $\Delta r = |r_{Status\ A}-r_{Status\ B}|$ is preferably greater than 85% of all peptide-to-peptide Δr.

Figure 15 shows a flow chart schematizing the above described procedural steps of an interaction of the Differential Network Module with the CAN Module according to the present invention allowing for the identification of peptides suitable to be used as marker panels using the peptide topology of a plurality of samples taken from at least two different experimental groups representing a status A and a status B. At step 170 a respective mass spectrum for each sample of said plurality of samples is provided, wherein signal intensity peaks correspond to potential peptides. Then the measures of correlation between the signal intensities of said potential peptides are computed at step 172 for each plurality of samples within each experimental group separately. Finally pairs of potential peptides are selected at step 174, which exhibit a difference in the degree of correlation between the different experimental groups above a certain threshold, thereby providing peptides which are suitable to be used as marker panels for diagnostic purposes to distinguish between status A and status B.

The results of the Differential Network Module allow statements about the different relations of peptides within samples of status A compared to status B as follows: If the difference of correlation coefficients of peptide I with peptide II in status A minus the corresponding correlation coefficient in status B is greater than a given threshold, signal coordinates of the peptide pairs, their mutual distance within the observed status A and status B or the degree of difference or combinations of the latter information are stored in a database or list. The Differential Network Module optionally provides the same visualization methods as the other modules, that means peptide coordinates and their relations can be represented as bullets connected by lines, respectively, as shown in Figure 6, and identified peptides can be reviewed via convenient connections to databases or lists containing supportive data resources.

Another use of this aspect of the present invention is the comparison of the molecular masses of peptides present in at least three samples, representing one or at least two different states, status A with corresponding samples and status B with corresponding samples. For example samples from individuals with a certain disease versus samples from individuals without that certain disease, samples from pregnant versus samples from non-pregnant individuals, samples from bacteria transformed with an expression vector versus samples from non-transformed bacteria, samples from yoghurt with a strong acidic taste versus samples from yoghurt with a mild acidic taste, etc. might be compared by computing the correlation measures of peptides present in these samples. The comparison of measurement parameters of a peptide within two samples corresponding to two different states A and B may also indicate that the peptide is present only in samples of state A but not in samples of state B. Also in this case the measurement parameters of this peptide in status A and status B possibly can be related by a measure of correlation. If at least two different peptides, e.g. peptide I and peptide II, are identified, the measurement values of the parameters for peptide I and peptide II can be combined. Using measurement values of at least three samples being representative of status A and three samples being representative of status B, a mathematical function can be computed. This mathematical function describes the correlation-network of peptide I and peptide II. It is possible to include more than two different peptides in one correlation-network, e.g. to include more than two different peptides in one mathematical function describing a correlation-network. The resulting mathematical function describes which combinations of measures of correlation of at least two peptides (peptide I and peptide II) allow to distinguish status A from status B.

Furthermore, another use of this aspect of the present invention comprises the automated identification of sets of peptides that allow a prediction of a status of a sample by a regression model. The invention detects relations between at least two peptides, where the relations are representative for a given status A. In a next step, a linear or non-linear regression model is set up that uses input parameters of the found peptides, such as their respective MALDI signal intensities, and that fits these input parameters to an end point parameter, such as the diagnosis (yes/no = 1/0), or that fits to another parameter of a peptide of this derived set.

In order to check whether a sample of unknown status is a member of the status A, the input parameters of these peptides from that sample are applied to the derived model. If the output value obtained from that sample deviates in the range as other samples from status A from an expected value obtained by means of the determined function, than this unknown sample can be considered to be from status A. Otherwise, the sample most likely has another status.

Marker Panel Network Module

According to the present invention the interaction of the Marker Panel Network Module with the fundamental CAN Module allows to identify peptides which independently from each other distinguish between a sample representing status A and a sample representing status B. For example a disease is caused by different factors such as inflammation and an increased heart beat rate. Each of these disease factors might result in altered concentrations of distinct peptides in for example blood plasma of the patient. If a panel of for example two peptide markers is used for diagnosis of the disease it would be useful if one of the peptide markers indicates inflammation and the other peptide marker indicates increased heart beat rate. The combination of these two markers would increase the specificity and sensitivity of the marker panel to detect the disease caused by a combination of inflammation and increased heart beat rate. The Marker Panel Network Module selects those potential peptides which are related to the disease but are most likely associated to different disease factors (in this hypothetical case inflammation and increased heart beat rate), since these peptide coordinates have a low measure of correlation to each other but both have a high correlation to the disease. Thus the specificity and sensitivity of a diagnostic test can be improved by combining these complementary peptide coordinates to a marker panel.

For example a disease 1 (status A) which is associated with inflammation has to be distinguished from another disease 2 (status B) which is not associated with inflammation. There are, for example, four peptides found, which distinguish disease 1 form disease 2. Peptide 1 and peptide 2 are fragments from the same protein, for example from TNF-alpha, peptide 3 is, for example, a fragment of IL-6 and peptide 4 is a fragment of an unknown protein. All of these four peptides differentiate between disease 1 and disease 2 by a measure of correlation, but peptide 1 and 2 correlate to each other, which is not surprising, as they originate from the same molecule (TNF-alpha). Additionally peptide 1 and peptide 3 correlate to each other, which is also not surprising, as TNF-alpha and IL-6 have similar pro-inflammatory functions. Consequently there are two groups of peptides, peptides 1, 2 and 3 belong to one group and peptide 4 represents the second group. To obtain a diagnostic test, with improved specificity and/or sensitivity combination of the detection of peptide 1 and 2 or 1 and 3 or 2 and 3 would not increase the specificity and/or sensitivity as much as combination of peptide 1 and 4 or 2 and 4 or 3 and 4 would do. This method allows to identify panels of peptides with additive or synergistic value (diagnostic, therapeutic, functional, etc.).

Figure 16 shows a flow chart schematizing the procedural steps of an interaction of the Marker Panel Network Module with the CAN Module according to the present invention allowing for the identification of peptides suitable to be used as marker panels using the peptide topology of a plurality of samples taken from at least two different experimental groups representing a status A and a status B. At step 180 a respective mass spectrum for each sample of said plurality of samples is provided, wherein signal intensity peaks correspond to potential peptides. Then potential peptides correlating with a parameter being representative of status A or status B are selected at step 182. Thereafter the measures of correlation between the signal intensities of said selected potential peptides for each plurality of samples are computed at step 184 and finally pairs of potential peptides which exhibit no correlation of their respective signal intensities above a certain threshold are selected at step 186, thereby providing potential peptides which are suitable to be used as complementing peptides in a marker panel for diagnostic purposes to distinguish between status A and status B.

In other words, the Marker Panel Network Module selects potential peptides which correlate with a parameter being representative for status A or status B. The Marker Panel Network Module then queries the Correlation Associated Network (CAN) Module for those pairs of selected peptide coordinates, which have a very low measure of correlation of their respective signal intensities to each other. The result are pairs of peptides which are related to the status A or B but not directly related to each other and can be combined for a marker panel to distinguish between status A and B. It is possible to combine two or more peptides to a marker panel.

The Differential Network Module described in the previous section discovers combinations of peptides, whose ratio of concentration indicate a certain state and deviations from that ratio indicate a different state. It is mandatory to measure the signal intensity (e.g. concentration) of both/any peptide to calculate said ratio. The relations between two peptides may be present only in state A, whereas the relations between the same two peptides may be different or absent in state B.

In contrast, any peptide found by the Marker Panel Network Module described in the present section could serve as a diagnostic marker alone, but a combination of both markers improves the sensitivity/specificity etc. of the diagnostic test. The members of a marker panel ideally should not correlate with each other in any of both states. If the members of a marker panel correlate with each other their combination most likely would not improve the sensitivity/specifity of the diagnosis.

Surrogate Network Module

The Surrogate Network Module relates to the identification of peptides (so called surrogate peptides) that can replace or complement established diagnostic or therapeutic peptides or peptides of other use. If for instance it is discovered that peptides correlate with known bioactive therapeutic peptides, these peptides might serve as surrogates for therapeutic measures or even may exhibit a higher/larger potency, efficacy, specificity, selectivity and/or less undesirable side effects. These kind of peptides can be found using the Surrogate Network Module in combination with the CAN Module according to the present invention by applying the steps shown in Figure 17. Initially a respective mass spectrum for each sample analyzed is provided, wherein signal intensity peaks correspond to potential peptides (step 190). Thereafter at step 192 measures of correlation between the signal intensity of a known peptide and the signal intensities of potential peptides are computed and finally those potential peptides are selected at step 194, which exhibit a degree of correlation with the known peptide above a certain threshold, thereby providing potential peptides suitable to replace or complement the known peptide. Two exemplary applications of the Surrogate Network Module are given below

For example a plasma sample is known to contain the peptide insulin and a potentially unknown peptide X within the same plasma sample correlates with the peptide insulin. In this case peptide X might have the same function as insulin, as its correlation measure indicates that it is related to insulin. The reason for this could be that peptide X is a derivative of insulin, for example a glycosylated form of insulin, or another peptide which is completely different from the amino acid sequence of insulin but which is involved in the same functional or metabolic cycles as insulin. In both cases peptide X could serve as an alternative to the use of insulin for example in treating diabetes. It might also turn out that peptide X in combination with insulin improves the therapeutic effect of insulin by itself.

In a further example a tissue sample of a prostate cancer patient contains the prostate-specific antigen (PSA) peptide, which is a known marker for prostate cancer. Another potentially unknown peptide Y is related by a correlation measure to the PSA peptide and consequently peptide Y might have the same diagnostic value as a biomarker for prostate cancer as the PSA peptide or the measurement of peptide Y might complement the prostate cancer diagnosis by PSA measurements.

Interaction of Modules

Though any of the modules described above can be used independently, any combination of these modules can be used and potentially can synergistically improve the result of one or more of the modules.

For example results of the Surrogate Network Module can be analyzed by the Sequence Network Module. In case the Surrogate Network Module yields peptide signals, which are not yet sequenced, a prediction of the sequence can give early hints for biological interpretation, thus accelerating validation processes of for example therapeutic or diagnostic peptides. However, a subsequent identification of these peptides by sequencing is recommended.

Results of the Differential Network Module can be analyzed with the Surrogate Network Module. If the Differential Network Module yields for example potential biomarkers, it is highly desirable to identify possible surrogate markers that show a similar behavior and therefore are of interest as well. Therefore a combination of the Surrogate Network Module with the Differential Network Module accelerates the discovery of novel therapeutic, diagnostic or other peptides and is highly synergistic.

Furthermore, results of the Differential Network Module can be analyzed with the Sequence Network Module. If the Differential Network Module yields peptide signals, which have not been sequenced yet, the prediction of the sequences of the unknown peptides can give early hints for biological interpretation, thus accelerating validation processes of potential therapeutic, diagnostic or other peptides. However, the later identification of these peptides by sequencing is recommended.

Examples

The following examples are intended to describe how the methods according to the present invention can be applied to real data. For the sake of a clarity only a limited number of exemplary measurement parameters are calculated and presented in the figures. However, as is readily observable by the person skilled in the art, the advantages of the methods according to the present invention become even more obvious when applied to large sets of data. On present computer systems commonly measures of correlation for data sets consisting of up to 6.000 potential peptides are commonly calculated and without undue effort data sets of up to 100.000 potential peptides can be analyzed by means of the methods according to the present invention.

Example 1

The basic CAN Module calculates to what extent a potential peptide for each individual potential peptide measured in a sample correlates to every other potential peptide in that sample. The CAN Module determines a network of correlations among the peptides which in case of some degree of correlation supposedly are related to each other for certain reasons such as a common precursor as the origin of the peptides or the same biological function of the different precursors of the correlating peptides.

In the present example the set of data, i.e. the data matrix, consists of 444.000 values comprising measurement parameters, in this case signal intensities, of 74 independent samples, each sample resulting in 6.000 peptide coordinates. The tables shown in Figures 18a, 18b list the corresponding raw data for four out of a total of 6.000 peptide coordinates. Four different methods to determine measures of correlation, namely, Spearman's rank order correlation, Pearson's product moment correlation, Kendall's rank correlation tau, and Minimal Spanning Tree (MST) diameter, are calculated for the three exemplified pairs of peptide coordinates comparing the peptide coordinate Fraction 54; m/z 2743.0 with three other peptide coordinates (Fraction 54; m/z 1371.5, Fraction 56; m/z 2927.2 and Fraction 20; m/z 1114.3) (see table shown in Figure 19). The definition of the threshold is an important step in the creation of Correlation Associated Networks and should be performed carefully as has been described in detail further above. In the data matrix 6.000 x 6.000 x 0.5 = 1.8 x 10^7 possible peptide-to-peptide pairs can be combined, and each of these pairs exhibits a certain correlation coefficient r. Figure 20 shows a plot of the probability of a peptide pair P(r) to have a certain correlation coefficient r. A value of r of zero or close to zero describes relations which are completely random, whereas values of r close to 1 or -1 describes relations which respectively correlate or anti-correlate very strongly. The more peptide pairs are tested for a correlation by means of measures of correlations, such as Spearman's rank order correlation coefficient, the more peptide pairs by chance correlate to some extent with each other. This means that a correlation coefficient regarded as informative and real has to pass a higher threshold value. It is recommended to perform a plot like in Figure 20 to estimate the information content of a given correlation coefficient. One curve (black circles) in this figure plots the likelihood (y-axis) for a given correlation coefficient (x-axis) for all peptide-to-peptide pairs from the said data matrix comprising 6.000 peptide coordinates. The other curve in Figure 20 marked by the white squares describes the likelihood of correlations occurring by chance.

Most probable true positive relations can be found where the area under the curve is small, while the maxima of the curves represent the correlations coefficients which are most likely false positive relations. In case Spearman's rank order correlation coefficient is chosen as measure of correlation and $|t_{threshold}| \geq 0.8$ is chosen as threshold for a definition of a peptide-to-peptide relation, the peptide coordinate Fraction 20; m/z 1114.3 is not related to the peptide coordinate Fraction 54; m/z 2743.0 (see table shown in Figure 19). In contrast, the peptide coordinates Fraction 54; m/z 1371.5, and F 56; m/z 2937.3 are highly related to the peptide with coordinates Fraction 54; m/z 2743.0 (see table shown in Figure 19). These peptide relations could pass through a filter and be stored in a local Valentina Database file.

Example 2

Assuming that one is interested in finding surrogate markers for Chromogranin A in hypothetical prostate cancer patients and that some of the 74 samples described above originated from healthy male persons and some samples originated from prostate cancer patients. Under the further assumption that a peptide originating from Chromogranin A, amino acids 97-131, had been identified, the Surrogate Network Module would now query the basic CAN Module for peptide coordinates that are highly related by a correlation measure with the hub-peptide Chromogranin A, 97-131. This could be done for example by defining that the Spearman's rank order correlation coefficient of peptide-to-peptide relations r has to comply with the relation $|r| \geq 0.67$. Then the Surrogate Network Module would instruct the CAN Module to query the Valentina Database, and report that there are about 14 peptide coordinates matching this condition. These peptide coordinates are searched in databases for any known peptide fitting to these coordinates. In this way it would be found that three peptides known from the database and present in the list of 14 peptides belong to the Chromogranin/Secretogranin family as illustrated in the table shown in Figure 21. The Surrogate Network Module would project the peptide coordinates of the related peptides and the hub peptide as bullets on a two-dimensional or three-dimensional plane, such as a peptide map fingerprint of a serum sample as shown in Figure 6. Relations between peptide coordinates are depicted as lines between the bullets. Lines can be selected by a computer pointing device such as a mouse and a small information window will pop up containing information about the kind of correlation measure and the value for the measure of correlation of the connected peptide coordinates is shown. The bullets can also be selected by a computer mouse click, and an information window will provide information about the peptide coordinate, and if this peptide coordinate has already been identified, then the name of the precursor peptide, the start- and stop position of the identified peptide will be provided by retrieval of a "Sequence Information Database" as exemplified at 56 in Figure 1. Also links to other databases such as Swiss-Prot and GeneCard are provided and/or other databases such as the Patent database of the USPTO can be queried for the search terms "name of the peptide" and "diagnostic". An internet browser window could display the results from the US-Patent Database. The visualization of peptide-to-peptide relations and convenient connection and access to internet and intranet resources by the Surrogate Network Module significantly increases the speed of data acquisition that is needed for an evaluation of the results. The example of Chromogranin A indicates that other peptides originating from members of the secretogranin-chromogranin family are automatically found by the CAN Module. These peptides are listed in the table shown in Figure 21 and can serve as a diagnostic marker for the prediction of the therapeutic success in the hypothetic prostate cancer patients.

Example 3

In an exemplary hypothetic serum dataset 48 samples are derived from patients before prostatectomy and 26 samples from patients after prostatectomy. For the Differential Network Module a correlation measure, e.g. the Spearman's rank order correlation coefficient r, between the peptides is calculated for samples from patients before prostatectomy and for samples from patients after prostatectomy separately. The correlation coefficient of Chromogranin A 97-131 and Secretogranin I 88-132 for all 74 samples is $r = 0.67$, for those patients before prostatectomy is $r = 0.23$ and for those after prostatectomy is $r = 0.97$ (see Figure 22). This shows that the peptides Chromogranin A 97-131 and Secretogranin I 88-132 obviously are much less related after prostatectomy than before. This also explains the loss of correlation for all patients. For the given example this means that Secretogranin I 88-132 is a potential surrogate marker for Chromogranin A 97-131 only before prostatectomy, thereafter the relation is broken. This would have a significant impact on the design of a clinical evaluation of Secretogranin I 88-132 as a surrogate marker for Chromogranin A, and could save enormous costs. Furthermore, the ratio of concentration of Chromogranin A 97-131 and Secretogranin I 88-132 is a diagnostic parameter itself. If the ratio deviates from 10/1 significantly, then a prostatectomy has been accomplished. Figure 22 exemplifies the use of the ratio of the signal intensities of Chromogranin A 97-131 and Secretogranin I 88-132 as a diagnostic parameter: The ratio of 10/1 is present in all samples from patients before prostatectomy. In samples after prostatectomy this ratio is not present, i.e. the Secretogranin I/Chromogranin A relation is "broken".

Example 4

This section exemplifies the identification of representative peptides, also called "landmark peptides" and also refers to the given data matrix of 74 observations of 6.000 peptide coordinates already discussed in a previous example.

Two peptide coordinates are considered as related if the Spearman's rank order correlation of their signal intensities is above r > 0.8. The number of relations k a respective peptide has with different peptide coordinate is shown in the second row of the table shown in Figure 18a. From all peptide coordinates, Fraction 54; m/z 2743.0 has the most relations, i.e. k = 20. Therefore, this peptide coordinate would be No. 1 in a prioritization list. Then, the signal variance of Fraction 54; m/z 2743.0 is removed from the signal intensities of the 20 related peptide coordinates, wherein Formulas 1, 2 and 3 are applied. Then the data of Fraction 54; m/z 2743.0 is removed from the data matrix. The tables shown in Figures 23a and 23b show the values given in the tables shown in Figures 18a and 18b after the variance of Fraction 54; m/z 2743.0 on the related peptide coordinates has been removed. This process is iterated to determine the next peptide coordinate as a candidate for the sequencing prioritization list, until the number of peptides to be sequenced has been reached.

Example 5

In this example, the signal intensities of four fictive peptide coordinates of 74 samples, their respective mass-to-charge ratio and their fraction numbers are given (see table shown in Figure 18a). The calculation is performed using five fictive peptide coordinates using as the 5th peptide coordinate F 53; m/z 2823.0. One of the five signal coordinates, the fictive peptide HP 25-48 in Fraction 54; m/z 2743.029, has already been identified, and guided by the rules for the Sequence Network Module, the identities of the four remaining, unknown peptides will be proposed.

The measure of correlation of the four unknown peptide coordinates with HP 25-48 has been calculated in the CAN Module by means of Spearman's rank order correlation coefficient:

r (HP 25-48 and F 20; m/z 1114.3) = +0.00
r (HP 25-48 and F 54; m/z 1371.5) = +0.92
r (HP 25-48 and F 56; m/z 2927.3) = +0.84
r (HP 25-48 and F 53; m/z 2823.0) = +0.87

As can be seen in Figures 24a to 24c and by the low correlation coefficient and MST diameter shown below, respectively, F 20; m/z 1114.3 is not related to HP 25-48, thus will not be hypothesized to be related to the HP precursor protein. The generation of proposals for this peptide coordinate stop at this point.

In the same manner, the MST diameter was calculated as a measure of correlation:

MST diameter (HP 25-48 and F 20; m/z 1114.3) = 29 (see Figure 25a)
MST diameter (HP 25-48 and F 54; m/z 1371.5) = 50 (see Figure 25b)
MST diameter (HP 25-48 and F 56; m/z 2927.3) = 38
MST diameter (HP 25-48 and F 53; m/z 2823.0) = 40 (see Figure 25c)

In contrast, peptide coordinates F 54; m/z 1371.5, F 53; m/z 2823.0 and F 56; m/z 2927.3 are highly related to HP 25-48 (see Figures 24b, 24c and Figures 25b, 25c). A proposal using the sequence of the precursor of the protein HP will be assigned to these peptide coordinates and the rules according to the Sequence Network Module of the present invention will be applied for sequence prediction.

Rule a determines whether the related peptide coordinate is a n-charged ion of HP 25-48. The calculation of $Mass_{Deviation}$ is exemplified with n = 1, 2, 3 or 4 and the mass-to charge ratios of F 54; m/z 1371.5 and F 56; m/z 2927.26 given in the table shown in Figure 26, using Formula 4. It is highly probable that F 56; m/z 1371.5 is a double charged ion of HP 25-48, as in the case of n = 2 $Mass_{Deviation} < Mass_{Threshold} = 0.5$, therefore it is proposed as HP 25-48^{2+}, i.e. the double charged ion of HP 25-48.

Rules b to i will now be applied to F 53; m/z 2823.0 and F 56; m/z 2927.3. Rule b assumes that the relation of the hub peptide P1 in fraction F 54; m/z 2743.029 of known identity with the unknown peptide P2 (peptide coordinate F 53; m/z 2823.0) is derived from a post-translational modification. In this case, the mass difference of the hub peptide P1 and the unknown peptide P2 $M_{DIFF} = |M_{P1}-M_{P2}| = 79.971$ might be caused by phosphorylation or sulphation (see table shown in Figure 9). Alignment of HP 25-48 with recognition sequence motifs of protein kinases, that are enzymes responsible for phosphorylation of proteins and peptides, identifies the sequence HP 35-37 to be "TYD", which is as a potential target of a hypothetic protein kinase HPKC. Therefore a proposal for F 53; m/z 2823.0 is HP 25-48 with one phosphorylation at the tyrosine residue on position 36 of the peptide HP 25-48.

As stated before, if the unknown peptide and the known hub peptide are related, it is hypothesized that the unknown peptide is derived from the same precursor protein and thus has the same precursor sequence as the known hub peptide. An algorithm systematically defines putative start and end positions, I an E, in the precursor sequence of the hub peptide P1 proposing a putative sequence fragment potentially derived from the precursor sequence, that could be the sequence of the unknown peptide P2 (see Figure 8b). Of course, the sum of masses of the amino acid residues, plus their amino- and carboxy-terminal ends and plus potential posttranslational modifications must match the measured m/z ratio M_{found} of the unknown peptide P2 within a given threshold T. The Mass of the putative sequence is calculated by summing up the masses of the amino acid residues comprising the putative sequence for P2 plus the mass of a hydrogen and a hydroxyl group. Exemplary values of masses applying Formula 5 are given in the tables shown in Figures 11 and 12.

With HP 25-48 as the hub peptide and P2 having the peptide coordinate Fraction 56; m/z 2927.3 the Sequence Network Module searches for possible sets of start and end positions in the protein precursor sequence of HP as defined in Figure 27, that have a deviation of mass lower than the threshold $T_{MASS} = 0.5$.

One possible combination is a start position at amino acid No. 25 and an end position at amino acid No. 50 of HP resulting in the potential peptide HP 25-50:
$n_D = 2$, $n_A = 4$, $n_H = 2$, $n_K=2$, $n_S = 1$, $n_E = 3$, $n_V = 2$, $n_R = 1$, $n_F = 1$, $n_L = 3$, $n_G = 1$, $n_I =1$, $n_N = 1$, $n_T = 1$, $n_Y = 1$ in Formula 5 results in $M_{CALC} = 2927.337$
This proposal is added to the list of proposals for P2.

The Sequence Network Module will now address the evaluation of the proposal HP 25-50 for P2 by applying rules c to i. In rule d, the chromatographic fraction of the proposed sequence F_{CALC} is estimated and compared with the found peptide coordinate of P2 (F_{FOUND}). If F_{CALC} deviates from F_{FOUND} by less than the threshold for fractionation ($T_{FRACTION}$) then the proposal is awarded with 2 model fitness points. If Formula 6, "Estimation of fraction number based on proposed sequence", is applied to HP 25-50, the calculated Fraction results in $F_{CALC} = 56$. As P2 HP 25-50 is found in fraction 56, the number of model fitness points for this proposal is increased by two points. Formula 6 was generated empirically from a mathematical model using data originating from liquor cerebrospinalis samples separated using a specific HPLC-column (as described in the patent application WO 03/048775 A2) using a specific software. Of course, for different types of samples and different separation methods other empirically determined models can be calculated in the same way.

Rule e rewards those proposals for P2, whose start-positions match the start positions of the hub peptide P1. In the case of HP 25-48 as the P1 hub peptide and HP 25-50 as the proposal for the related peptide P2, the proposal HP 25-50 will be rewarded with 3 model fitness points.

Rule f rewards those proposals for P2, whose end-positions equal the end-position of the hub peptide P1. This is not the case with HP 25-50 as a proposal, therefore this rule does not increase the model fitness points of this proposal for P2.

Rule g will increase the model fitness points of the proposal HP 25-50 by three points as the start position 25 is preceded by the amino acid sequence "R-R" (written in 1-letter amino acid code). The sequence "R-R" is a recognition site of prohormone convertases, which commonly cleave after the second "R". In addition, rule g will increase the model fitness points for this proposal by another 3 points, as the "D-A" sequence is one of the preferred starts for peptide sequences present in liquor cerebrospinalis. Further sites of frequent proteolytic cleavage sites at start positions awarded by rule f are well known in the art.

Rule g assumes that the unknown peptide P2 is a product of N- or C-terminal proteolysis of the known hub peptide P1 or vice versa. The mass difference of P1 and P2 $M_{DIFF} = |M_{P1}-M_{P2}|$ is determined and aligned with the masses of the amino acids preceding and following the start- and end positions of P2 in the precursor sequence HP. In the example of HP 25-48 as P1 and HP 28-50 as P2 the mass difference is $M_{DIFF} = 184.2$ and can be explained by the amino acids "I-A" ($M_I + M_A = 184.2$) which are following the end position of P1. Therefore P2 fits the model and the model fitness points for this proposal for P2 are increased by 3 points.

Obviously, rules c to i can be examined in any order, and rules can be left out for biological considerations, but still any combinations and any omissions of these rules are within the scope of this invention.

The process described above can be repeated for all unknown peptides coordinates P2, which are related with HP 25-48.

While this invention has been described with reference to preferred embodiments, it will be understood by those skilled in the art that various changes or modifications in form and detail may be made without departing from the scope of the invention as defined in the following claims.

For example it is readily apparent that the present invention can advantageously be utilized basically with all kinds of samples potentially containing peptides, such as samples from animals, plants, fungi, humans, parasites, microorganisms, such as bacteria, yeasts, viruses, and the like, samples from food or other agricultural materials such as meat, milk, grain, vegetables, wool, cotton, silk, samples from cosmetic products or other products containing peptides such as cleaning agents (often containing proteolytic enzymes), etc. Samples for example can be plasma, serum, hemo-filtrate, whole blood, blood cells, tissues samples, in vitro grown cells, cell culture supernatants, urine, cerebrospinal fluid, lymph fluid, sputum, tear fluid, ascites, preparations of cell organelles, tissue homogenate or homogenates of a virus, a microorganism, a parasite, a multi-cellular organism, an animal, a fungus or a plant and the like or combinations thereof. Examples of combinations are in vitro cultured cells infected with a microorganism or treated with pharmaceutical substances, tissue samples of humans infected with a microorganism, food products containing microorganisms, tissue culture supernatants of cells treated with peptides or mixtures of peptides present in food or cosmetic products, and the like.

New European Patent Application
BIOVISION AG
Our Ref.: 412-4

CLAIMS

1. A method of providing a representative, non-redundant overview of the peptide content of a sample type by analyzing a plurality of samples using its peptide topology, wherein the method comprises the steps of:

a) providing a respective mass spectrum for each sample of said plurality of samples, wherein signal intensity peaks correspond to potential peptides,

b) computing the measures of correlation between the signal intensities of said potential peptides,

c) grouping potential peptides together, which exhibit a degree of correlation among each other above a certain threshold, thereby providing a plurality of correlation associated networks of potential peptides, and

d) assigning one representative potential peptide out of each correlation associated network as a representative peptide to said correlation associated network of said sample type.

2. A method for predicting the sequence of peptides using the peptide topology of a plurality of samples containing a peptide having a known precursor, wherein the method comprises the steps of:

a) providing a respective mass spectrum for each sample of said plurality of samples, wherein signal intensity peaks correspond to potential peptides,

b) identifying said peptide having a known precursor using the mass of said peptide, wherein the sequence of the known precursor is known,

c) computing the measures of correlation between the signal intensity of said peptide having a known precursor and the signal intensities of the other potential peptides,

d) selecting potential peptides, which exhibit a degree of correlation with said peptide having a known precursor above a certain threshold, and

e) predicting the sequence of the potential peptides by matching masses of putative fragments of the sequence of the known precursor with the masses of the potential peptides correlating with said peptide having a known precursor.

3. A method for predicting the sequence of peptides using the peptide topology of a plurality of samples containing a peptide with a known sequence, wherein the method comprises the steps of:

a) providing a respective mass spectrum for each sample of said plurality of samples, wherein signal intensity peaks correspond to potential peptides,

b) identifying a peptide with a known sequence using its mass,

c) computing the measures of correlation between the signal intensity of said known peptide and the signal intensities of the potential peptides,

d) selecting potential peptides, which exhibit a degree of correlation with the known peptide above a certain threshold,

e) computing the mass differences between each of the potential peptides and the known peptide, and

f) predicting the sequence and/or the biologically, chemically or physically modified sequence of the potential peptides by using data about mass differences caused by biological, chemical or physical processes matching the mass differences determined in step e).

4. A method for identifying peptides suitable to be used as marker panels using the peptide topology of a plurality of samples taken from at least two different experimental groups representing a status A and a status B, wherein the method comprises the steps of:

a) providing a respective mass spectrum for each sample of said plurality of samples, wherein signal intensity peaks correspond to potential peptides,

b) computing the measures of correlation between the signal intensities of said potential peptides for each plurality of samples within each experimental group separately, and

c) selecting pairs of potential peptides, which exhibit a difference in the degree of correlation between the different experimental groups above a certain threshold, thereby providing peptides which are suitable to be used as marker panels for diagnostic purposes to distinguish between status A and status B.

5. A method for identifying peptides suitable to be used as marker panels using the peptide topology of a plurality of samples taken from at least two different experimental groups representing a status A and a status B, wherein the method comprises the steps of:

a) providing a respective mass spectrum for each sample of said plurality of samples, wherein signal intensity peaks correspond to potential peptides,

b) selecting potential peptides correlating with a parameter being representative of status A or status B,

c) computing the measures of correlation between the signal intensities of said selected potential peptides for each plurality of samples, and

d) selecting pairs of potential peptides which exhibit no correlation of their respective signal intensities above a certain threshold, thereby providing potential peptides which are suitable to be used as complementing peptides in a marker panel for diagnostic purposes to distinguish between status A and status B.

6. A method for identifying peptides suitable as a surrogate for a known peptide using the peptide topology of a plurality of samples, wherein the method comprises the steps of:

a) providing a respective mass spectrum for each sample of said plurality of samples, wherein signal intensity peaks correspond to potential peptides,

b) computing the measures of correlation between the signal intensity of said known peptide and the signal intensities of potential peptides, and

c) selecting potential peptides, which exhibit a degree of correlation with said known peptide above a certain threshold, thereby providing potential peptides suitable as a surrogate for said known peptide.

7. The method according to any one of claims 1 to 3 or 6, where a plurality of minimal spanning tree diameters is computed as a measure of correlation using the signal intensity of said potential peptides in said samples, wherein the selection of potential peptides is done by using minimal spanning tree diameter threshold, wherein the minimal spanning tree diameter for an association of two potential peptides has to be above an adjustable threshold of at least 0.425 times the number of samples.

8. The method according to claims 4 or 5, where a plurality of minimal spanning tree diameters is computed as a measure of correlation using the signal intensity of said potential peptides in said samples, wherein the selection of pairs of potential peptides is done by using minimal spanning tree diameter threshold, wherein the difference between the minimal spanning tree diameter found in the said different experimental groups is above an adjustable threshold of at least 0.1 times the number of samples.

9. The method according to any one of the preceding claims, wherein the method comprises the additional step of at least one fractionating step of said samples prior to providing the mass spectra of said samples and wherein at least one fraction of said samples is used for providing said mass spectra.

10. The method according to any one of the preceding claims using at least one measure of correlation selected from the group consisting of “Pearson Product-Moment Correlation Coefficient”, “Spearman’s rank order Correlation Coefficient”, “Kendall’s Tau”, “Kendall’s Coefficient of Concordance”, “Goodman and Kruskal’s Gamma” and “Minimal Spanning Tree diameters”.

11. The method according to any one of the preceding claims using at least one method for calibrating the mass spectrometric data selected from the group consisting of “Simple Offset Correction”, “2-Point Baseline Correction“, “Multi-Point Baseline Correction“, “Interactive Polynomial Baseline Correction“, “Function Fit Baseline Correction“, and “GIFTS (Auto Leveling Method) Baseline Correction”.

12. The method according to any one of the preceding claims using at least one method for identifying outlier samples selected from the group consisting of “Principal Component Analysis”, “multivariate calibration partial least-squares”, and “Replicator Neural Networks”.

13. The method according to any one of the preceding claims, wherein the calculation of the measures of correlation is repeated at least once using the peptide coordinates resulting from the previous round of calculations of measures of correlation, thereby providing the measures of correlation of 2nd or higher order neighborhood.

14. The method according to any one of the preceding claims using additional coordinates besides the mass selected from the group consisting of fraction number, elution time, retention time, protein chip coordinates, peptide concentration, enzyme activities, structural properties, chemical properties and biological properties.

15. The method according to any one of the preceding claims, wherein MALDI mass spectrometry or ESI mass spectrometry is used to generate the mass spectra.

16. The method according to any one of the preceding claims, wherein the samples or groups of samples are homogeneous.

17. The method according to any one of the preceding claims, wherein the computation of measures of correlation is done in advance prior to the analysis to accelerate the speed of the analysis using pre-determined values the measures of correlation.

18. The method according to any one of the preceding claims, wherein the necessary sequence information is provided by manual input or automatically queried from a database.

19. The method according to any one of the preceding claims, wherein the corresponding results are automatically combined with data from other sources chosen from the group consisting of sequence databases, patent databases, literature databases, medical databases, 3D structure databases, databases containing information about enzyme recognition sites, postranslational modifications, genetic polymorphisms, clinical trials.

20. The method according to any one of the preceding claims, wherein at least one step of data processing or data supply is done using a remote computer system and wherein the user is connected via an internet, intranet or other network to the remote computer system.

21. A digital computer system programmed to perform a method according to any one of the preceding claims.

22. A computer readable medium storing a computer program implementing a method according to any one of claims 1 to 20.

New European Patent Application
BIOVISION AG
Our Ref.: 412-4

ABSTRACT

The invention provides methods and a system implementing these methods for identifying and characterizing peptides and their functional relationships by use of measures of correlation. These methods are based on the interaction of a Correlation Associated Network Module with several Application Modules including a Sequence Network Module, a Differential Network Module, a Marker Panel Network Module and a Surrogate Network Module allowing e.g. the provision of a representative overview of the peptide content of biological samples, the prediction of peptide sequences, the identification of peptides suitable to be used as marker panels and the identification of peptides suitable as surrogates for a known peptide.

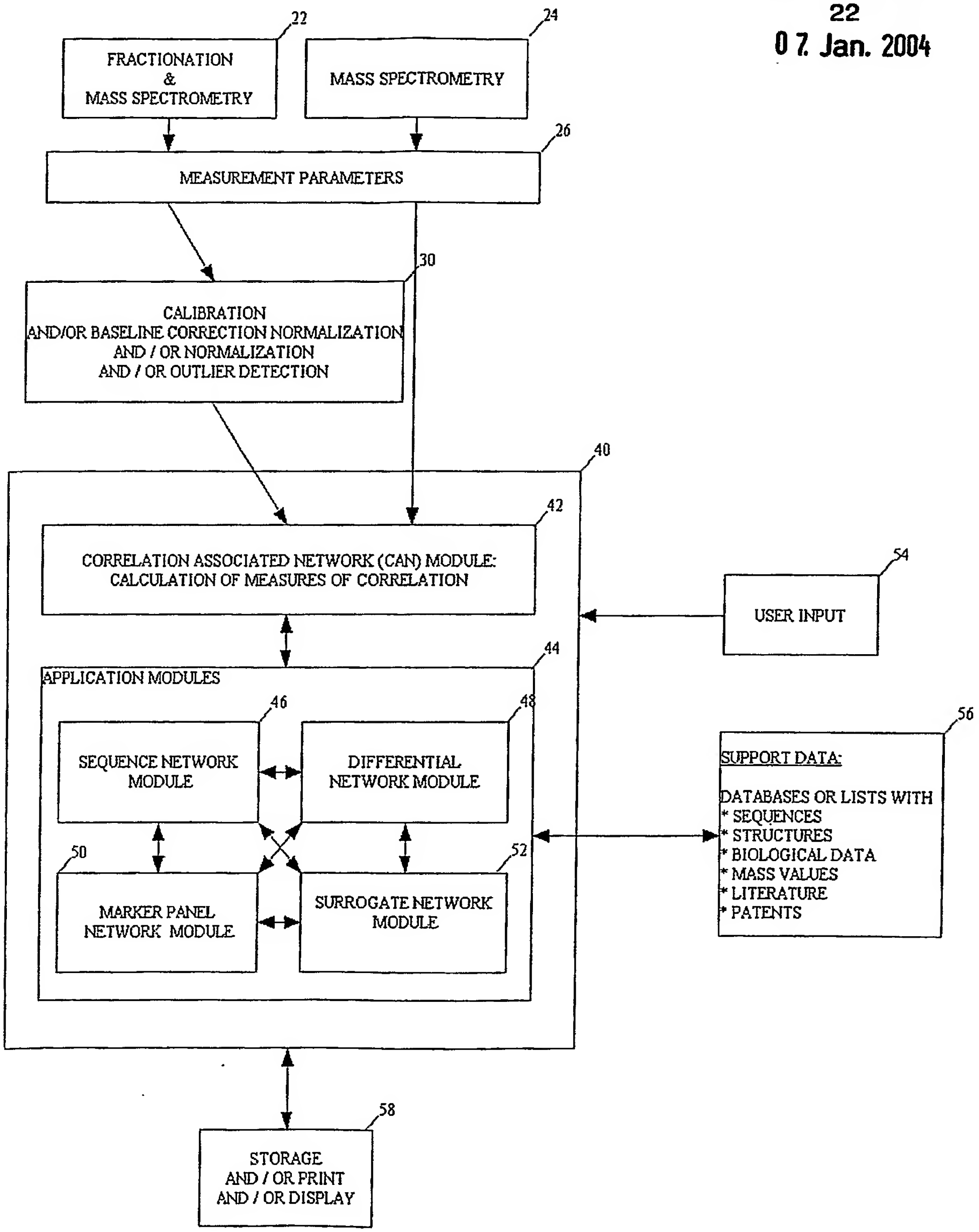

22
FRACTIONATION
&
MASS SPECTROMETRY
24
MASS SPECTROMETRY
26
MEASUREMENT PARAMETERS
30
CALIBRATION
AND/OR BASELINE CORRECTION NORMALIZATION
AND / OR NORMALIZATION
AND / OR OUTLIER DETECTION
40
42
CORRELATION ASSOCIATED NETWORK (CAN) MODULE:
CALCULATION OF MEASURES OF CORRELATION
54
USER INPUT
44
APPLICATION MODULES
46
SEQUENCE NETWORK MODULE
48
DIFFERENTIAL NETWORK MODULE
50
MARKER PANEL NETWORK MODULE
52
SURROGATE NETWORK MODULE
56
SUPPORT DATA:
DATABASES OR LISTS WITH
* SEQUENCES
* STRUCTURES
* BIOLOGICAL DATA
* MASS VALUES
* LITERATURE
* PATENTS
58
STORAGE
AND / OR PRINT
AND / OR DISPLAY

Fig. 1

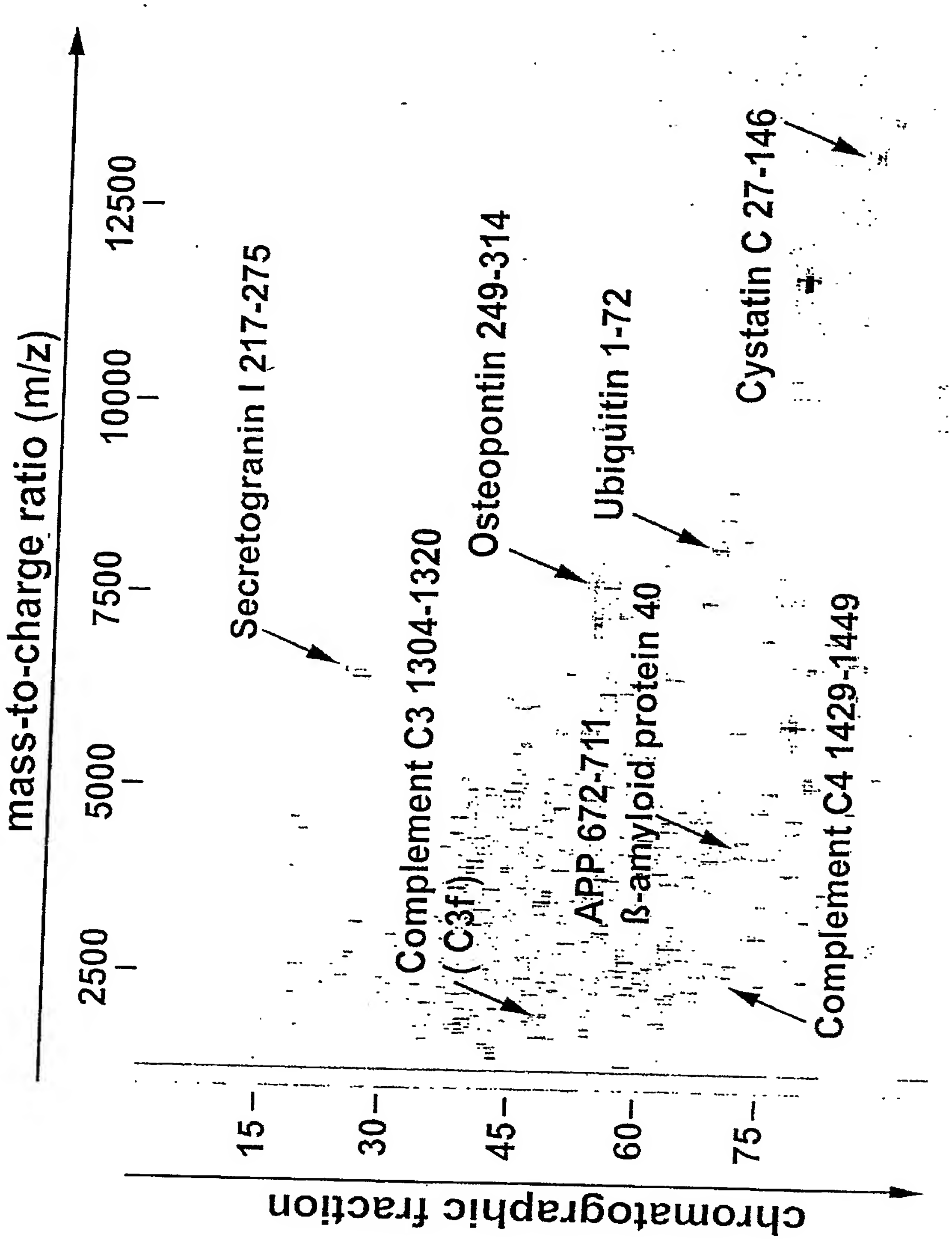

mass-to-charge ratio (m/z)
2500
5000
7500
10000
12500
Secretogranin I 217-275
Complement C3 1304-1320
(C3f)
Osteopontin 249-314
APP 672-711
ß-amyloid protein 40
Ubiquitin 1-72
Cystatin C 27-146
Complement C4 1429-1449
15
30
45
60
75
chromatographic fraction

Fig. 2

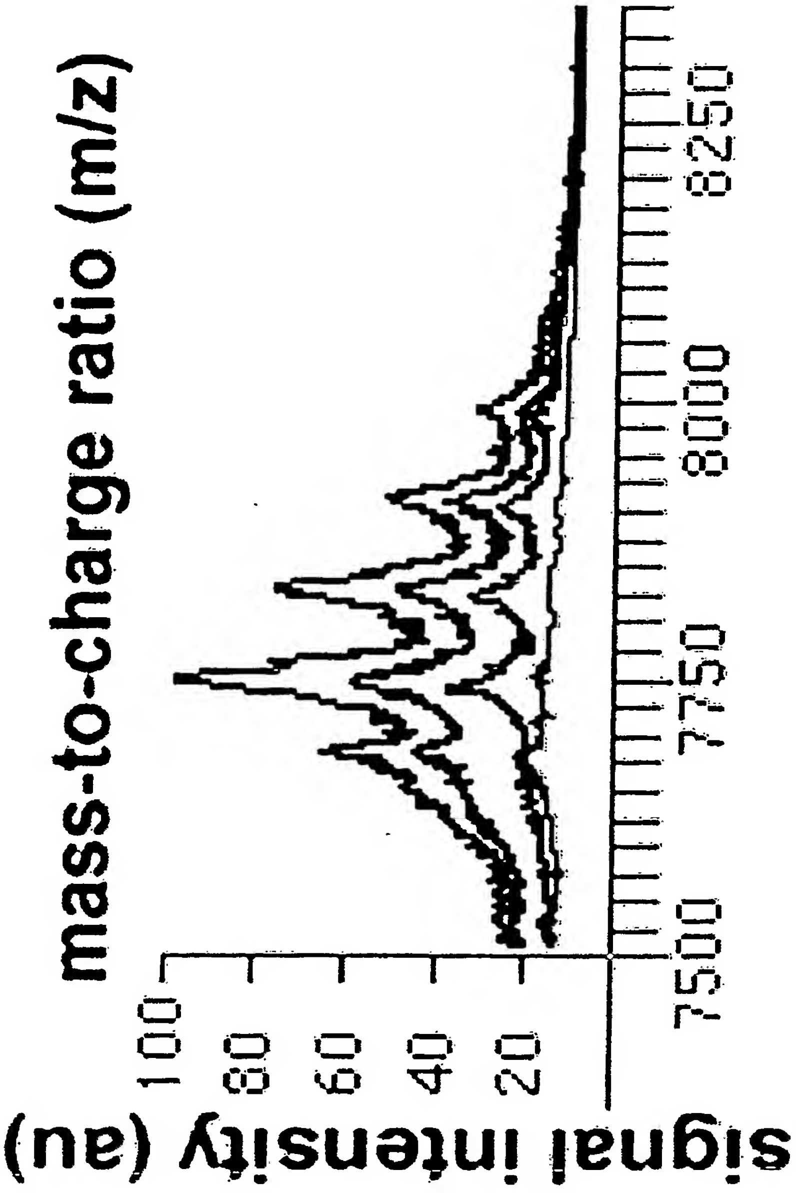

mass-to-charge ratio (m/z)
signal intensity (au)
100
80
60
40
20
7500
7750
8000
8250

Fig. 3

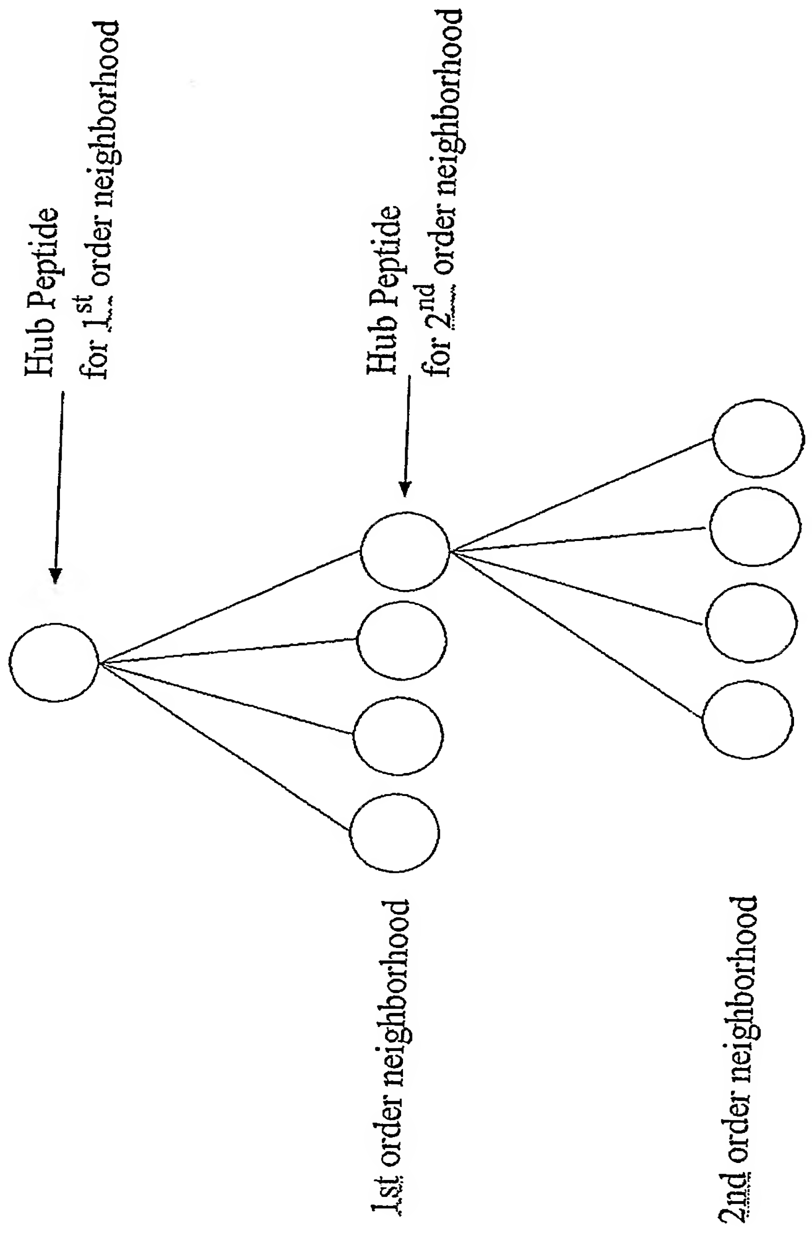

Hub Peptide
for 1st order neighborhood
Hub Peptide
for 2nd order neighborhood
1st order neighborhood
2nd order neighborhood

Fig. 4

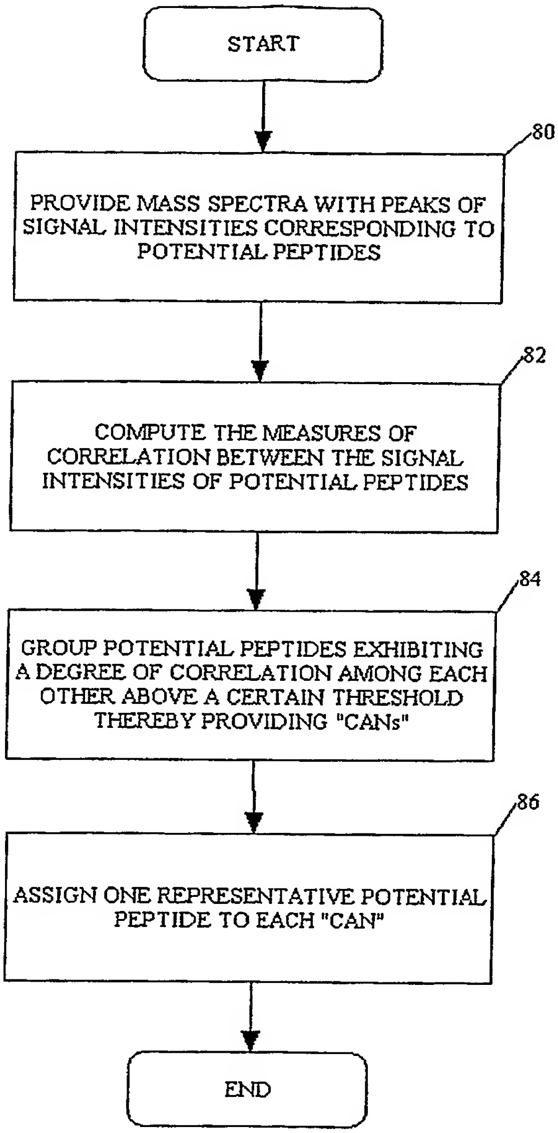

START
80
PROVIDE MASS SPECTRA WITH PEAKS OF SIGNAL INTENSITIES CORRESPONDING TO POTENTIAL PEPTIDES
82
COMPUTE THE MEASURES OF CORRELATION BETWEEN THE SIGNAL INTENSITIES OF POTENTIAL PEPTIDES
84
GROUP POTENTIAL PEPTIDES EXHIBITING A DEGREE OF CORRELATION AMONG EACH OTHER ABOVE A CERTAIN THRESHOLD THEREBY PROVIDING "CANs"
86
ASSIGN ONE REPRESENTATIVE POTENTIAL PEPTIDE TO EACH "CAN"
END

Fig. 5

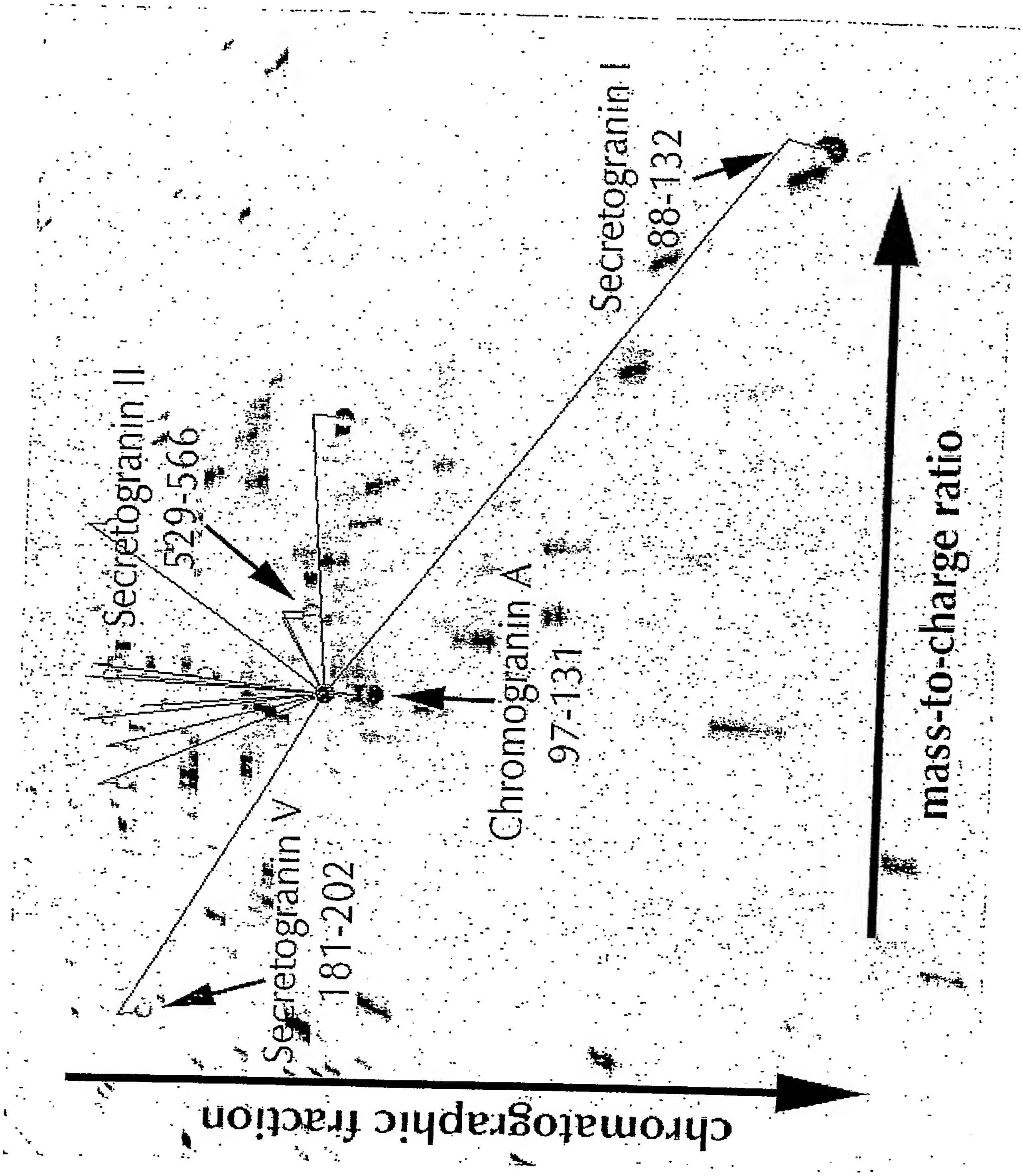

Secretogranin II
529-566
Secretogranin I
88-132
Chromogranin A
97-131
Secretogranin V
181-202
mass-to-charge ratio
chromatographic fraction

Fig. 6

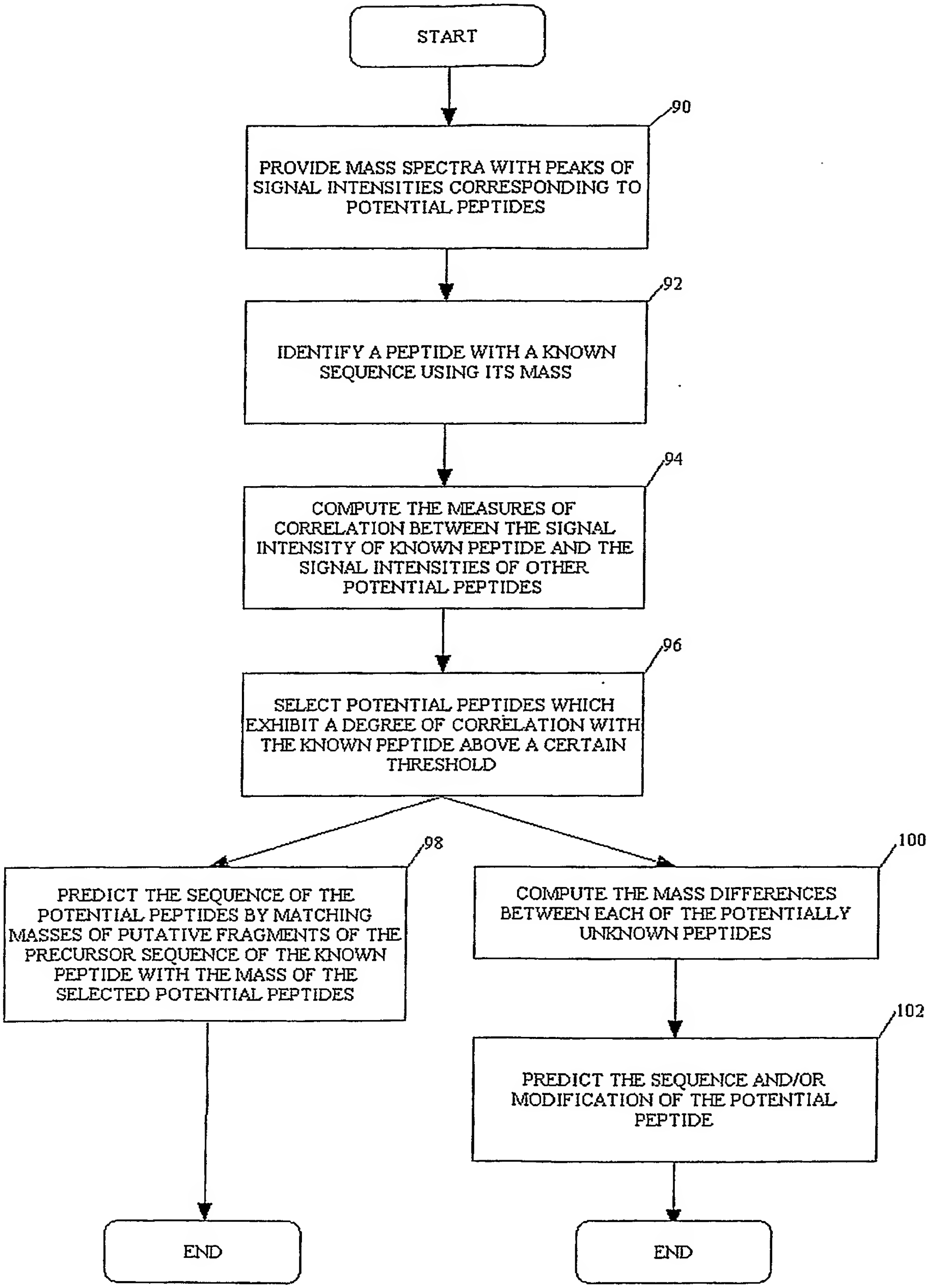

START
90
PROVIDE MASS SPECTRA WITH PEAKS OF SIGNAL INTENSITIES CORRESPONDING TO POTENTIAL PEPTIDES
92
IDENTIFY A PEPTIDE WITH A KNOWN SEQUENCE USING ITS MASS
94
COMPUTE THE MEASURES OF CORRELATION BETWEEN THE SIGNAL INTENSITY OF KNOWN PEPTIDE AND THE SIGNAL INTENSITIES OF OTHER POTENTIAL PEPTIDES
96
SELECT POTENTIAL PEPTIDES WHICH EXHIBIT A DEGREE OF CORRELATION WITH THE KNOWN PEPTIDE ABOVE A CERTAIN THRESHOLD
98
PREDICT THE SEQUENCE OF THE POTENTIAL PEPTIDES BY MATCHING MASSES OF PUTATIVE FRAGMENTS OF THE PRECURSOR SEQUENCE OF THE KNOWN PEPTIDE WITH THE MASS OF THE SELECTED POTENTIAL PEPTIDES
100
COMPUTE THE MASS DIFFERENCES BETWEEN EACH OF THE POTENTIALLY UNKNOWN PEPTIDES
102
PREDICT THE SEQUENCE AND/OR MODIFICATION OF THE POTENTIAL PEPTIDE
END
END

Fig. 7

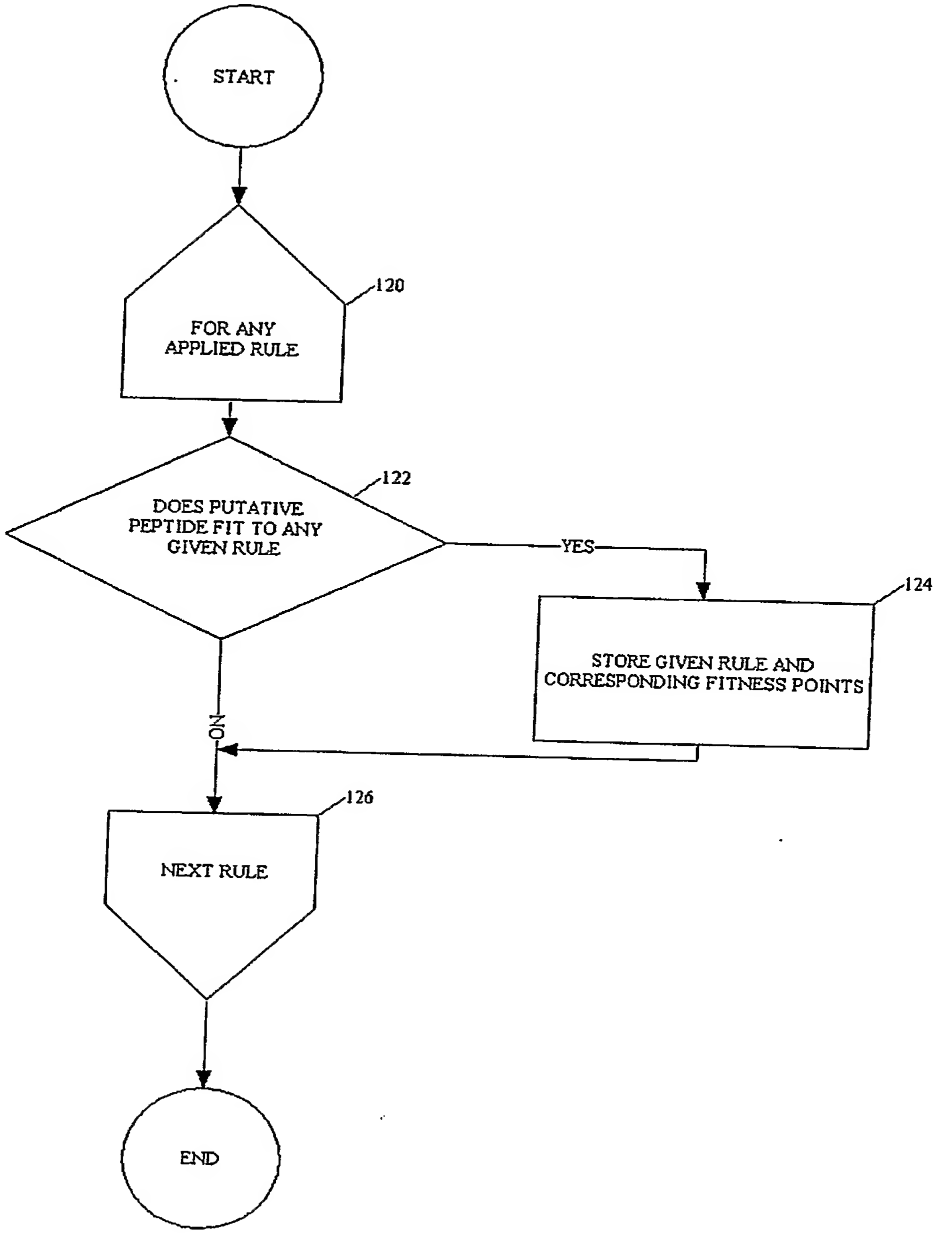

START
FOR ANY APPLIED RULE
120
DOES PUTATIVE PEPTIDE FIT TO ANY GIVEN RULE
122
YES
STORE GIVEN RULE AND CORRESPONDING FITNESS POINTS
124
NO
NEXT RULE
126
END

Fig. 8a

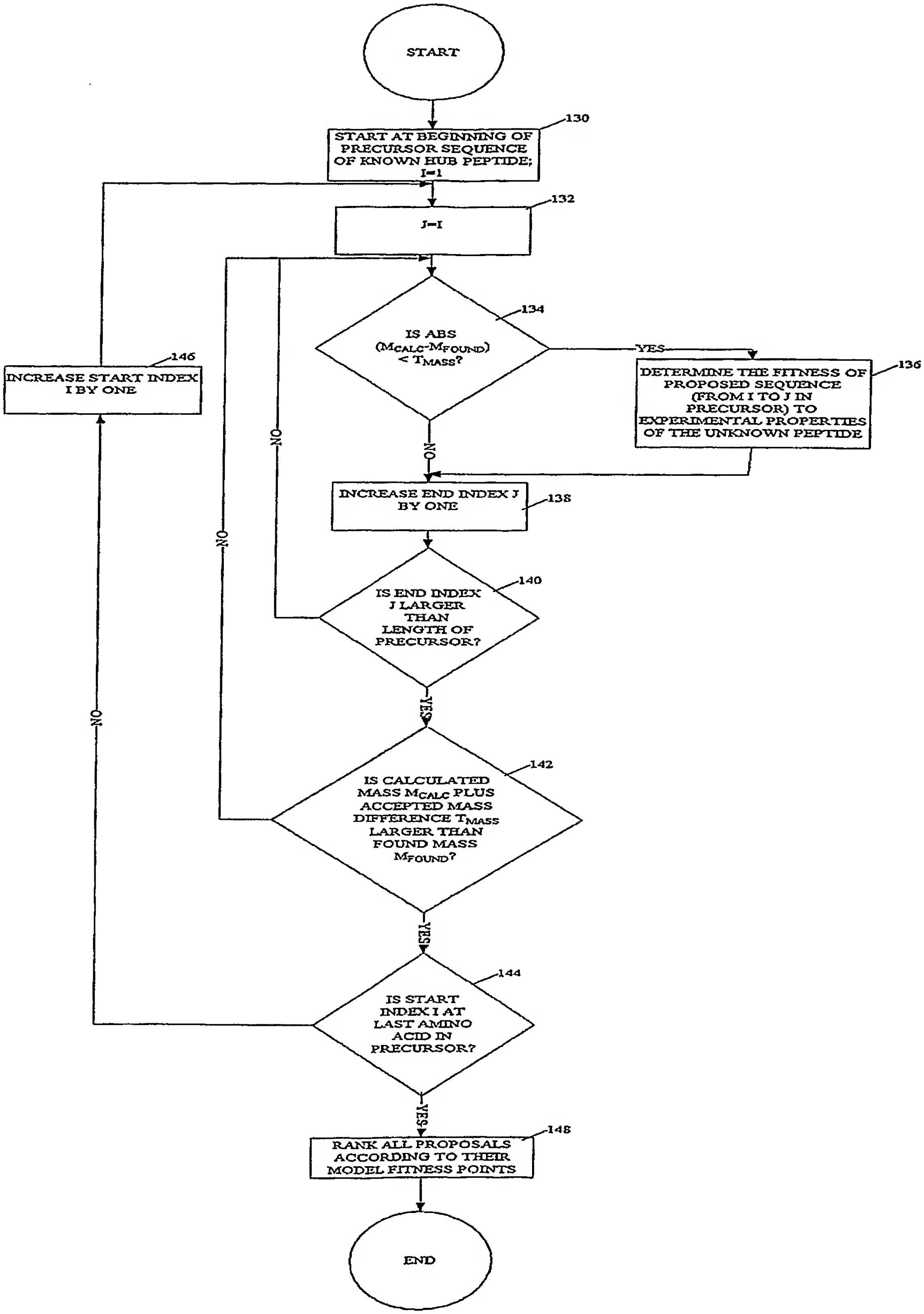

START
START AT BEGINNING OF PRECURSOR SEQUENCE OF KNOWN HUB PEPTIDE; I=1
130
J=I
132
IS ABS (M_{CALC}-M_{FOUND}) < T_{MASS}?
134
YES
DETERMINE THE FITNESS OF PROPOSED SEQUENCE (FROM I TO J IN PRECURSOR) TO EXPERIMENTAL PROPERTIES OF THE UNKNOWN PEPTIDE
136
NO
INCREASE END INDEX J BY ONE
138
IS END INDEX J LARGER THAN LENGTH OF PRECURSOR?
140
NO
YES
IS CALCULATED MASS M_{CALC} PLUS ACCEPTED MASS DIFFERENCE T_{MASS} LARGER THAN FOUND MASS M_{FOUND}?
142
NO
YES
IS START INDEX I AT LAST AMINO ACID IN PRECURSOR?
144
NO
INCREASE START INDEX I BY ONE
146
YES
RANK ALL PROPOSALS ACCORDING TO THEIR MODEL FITNESS POINTS
148
END

Fig. 8b

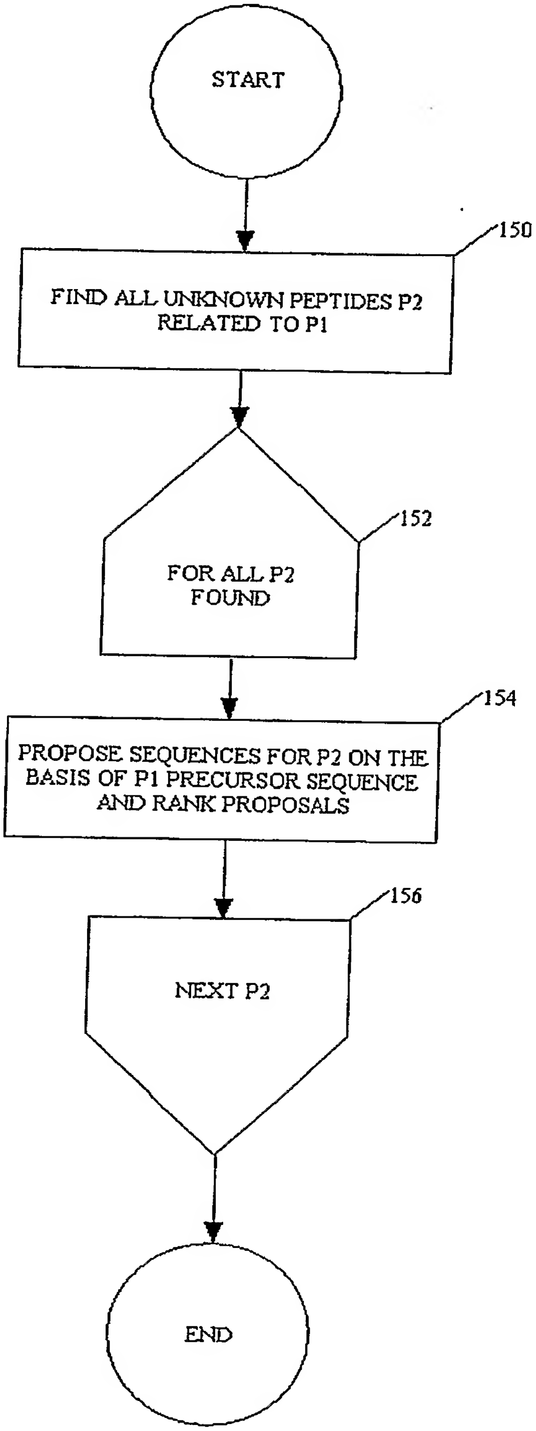

START
150
FIND ALL UNKNOWN PEPTIDES P2 RELATED TO P1
152
FOR ALL P2 FOUND
154
PROPOSE SEQUENCES FOR P2 ON THE BASIS OF P1 PRECURSOR SEQUENCE AND RANK PROPOSALS
156
NEXT P2
END

Fig. 8c

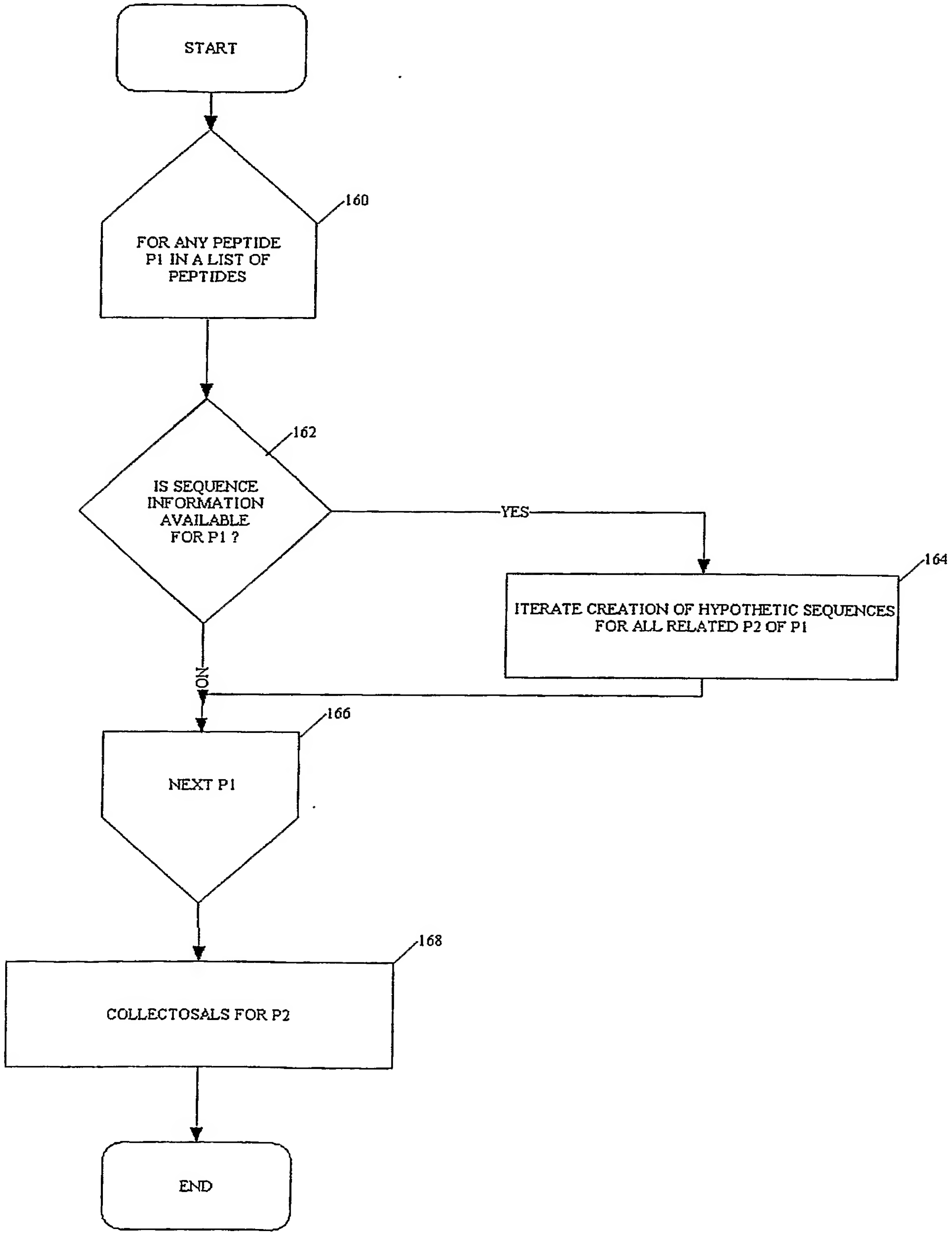

START
FOR ANY PEPTIDE P1 IN A LIST OF PEPTIDES
160
IS SEQUENCE INFORMATION AVAILABLE FOR P1 ?
162
YES
ITERATE CREATION OF HYPOTHETIC SEQUENCES FOR ALL RELATED P2 OF P1
164
NO
NEXT P1
166
COLLECTOSALS FOR P2
168
END

Fig. 8d

Modification	Monoisotopic Mass	Average Mass
4-Phosphopantetheine	339.0780	339.3294
S-Adenosylation	329.0525	329.2091
Acetylation	42.0106	42.0373
ADP-ribosylation (from NAD)	541.0611	541.3052
Biotinylation (amide bond to lysine)	226.0776	226.2994
Carboxylation of Asp and Glu	43.9898	44.0098
C-terminal amide formed from Gly	-0.9840	-0.9847
Cysteinylation	119.0041	119.1442
Deamidation of Asn and Gln	0.9840	0.9847
Deoxyhexoses (Fuc, Rha)	146.0579	146.1430
Disulphide bond formation	-2.0157	-2.0159
Farnesylation	204.1878	204.3556
Formylation	27.9949	28.0104
Geranylation	272.2504	272.4741
Glutathionylation	305.0682	305.3117
Hexosamines (GalN, GlcN)	161.0688	161.1577
Hexoses (Fru, Gal, Glc, Man)	162.0528	162.1424
Homoserine formed from Met by CNBr treatment	-29.9928	-30.0935
Hydroxylation	15.9949	15.9994
Lipoic acid (amide bond to lysine)	188.0330	188.3147
Methylation	14.0157	14.0269
Myristoylation	210.1984	210.3598
N-acetylhexosamines (GalNAc, GlcNAc)	203.0794	203.1950
N-acetylneuraminic acid (Sialic acid, NeuAc, NANA, SA)	291.0954	291.2579
N-glycolylneuraminic acid (NeuGc)	307.0903	307.2573
Oxidation of Met	15.9949	15.9994
Palmitoylation	238.2297	238.4136
Pentoses (Ara, Rib, Xyl)	132.0423	132.1161
Phosphorylation	79.9663	79.9799
Proteolysis of a single peptide bond	18.0106	18.0153
Pyridoxal phosphate (Schiff Base formed to lysine)	231.0297	231.1449
Pyroglutamic acid formed from Gln	-17.0265	-17.0306
Stearoylation	266.2610	266.4674
Sulphation	79.9568	80.0642

Fig. 9

Motif	Enzyme/ Reaction	Mass difference(average mass)
W	photochemical	+16
W	photochemical	+32
W	photochemical	+4
[ST]-X-[RK]	Protein kinase C	+79.9799

Fig. 10

Symbols		Monoisotopic Mass	Average Mass
Gly	G	57.02146	57.05
Ala	A	71.03711	71.08
Ser	S	87.03202	87.08
Pro	P	97.05276	97.12
Val	V	99.06841	99.07
Thr	T	101.0476	101.1
Cys	C	103.0091	103.1
Leu	L	113.0840	113.2
Ile	I	113.0840	113.2
Asn	N	114.0429	114.1
Asp	D	115.0269	115.1
Gln	Q	128.0585	128.1
Lys	K	128.0949	128.2
Glu	E	129.0425	129.1
Met	M	131.0404	131.2
His	H	137.0589	137.1
Phe	F	147.0684	147.2
Arg	R	156.1011	156.2
Tyr	Y	163.0633	163.2
Trp	W	186.0793	186.2

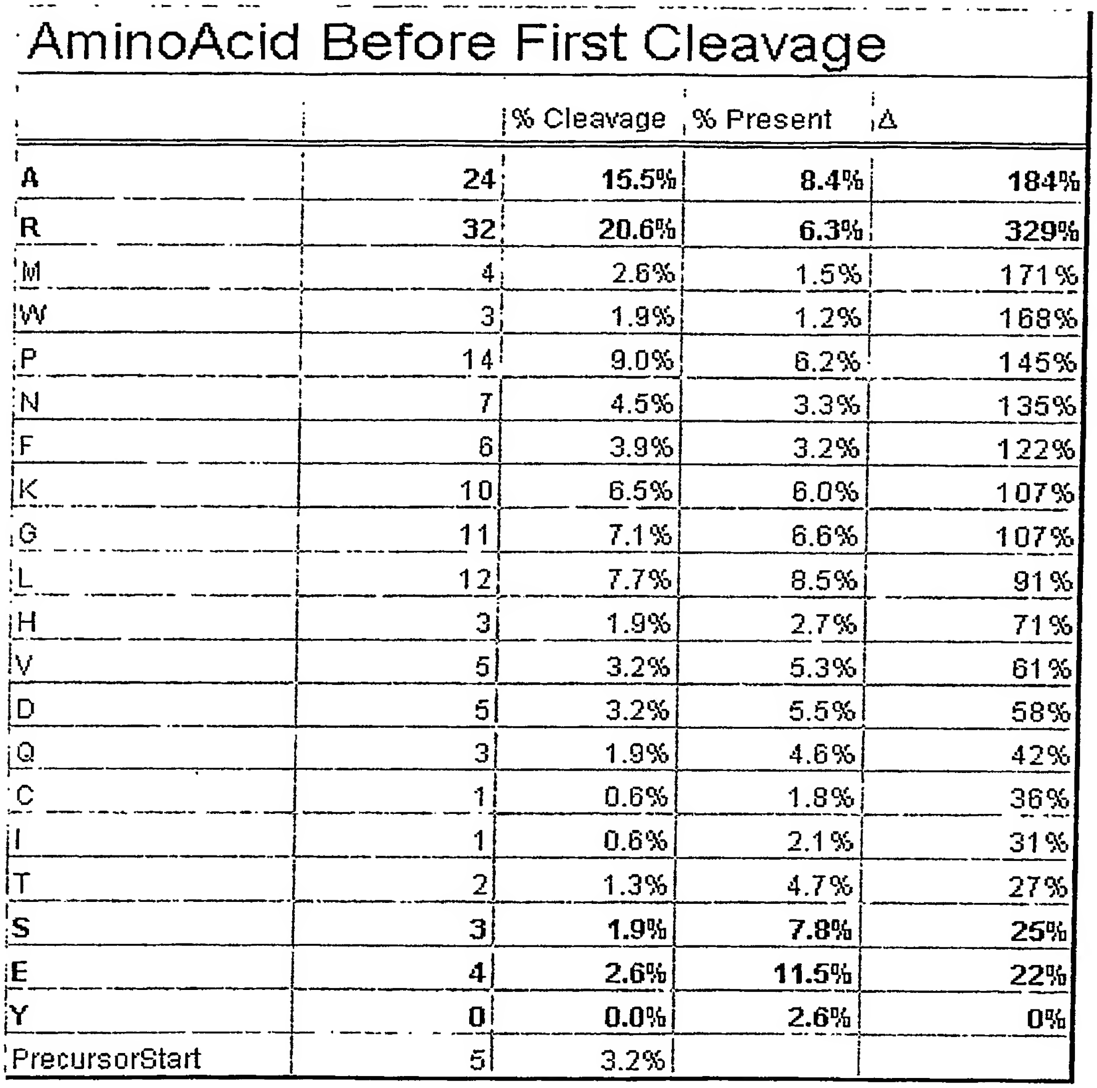
Fig. 11

	Composition	Monoisotopic Mass	Average Mass
N-Terminal Groups			
Hydrogen	H	1.00782	1.0079
N-Formyl	HCO	29.00274	29.0183
N-Acetyl	CH_3CO	43.01839	43.0452
C-Terminal Groups			
Free acid	OH	17.00274	17.0073

Fig. 12

Additional Amino acid	+/- Fraction numbers
E, Glutamic acid	+ 0.47
F, Phenylalanine	+ 5.54
H, Histidine	- 3.82
I, Isoleucine	+ 2.86
K, Lysine	- 1.72
L, Leucine	+ 5.15
Q, Glutamine	- 0.85
S , Serine	- 0.45
V, Valine	+ 2.2
W, Tryptophane	+ 5.35
Y, Tyrosine	+ 2.92

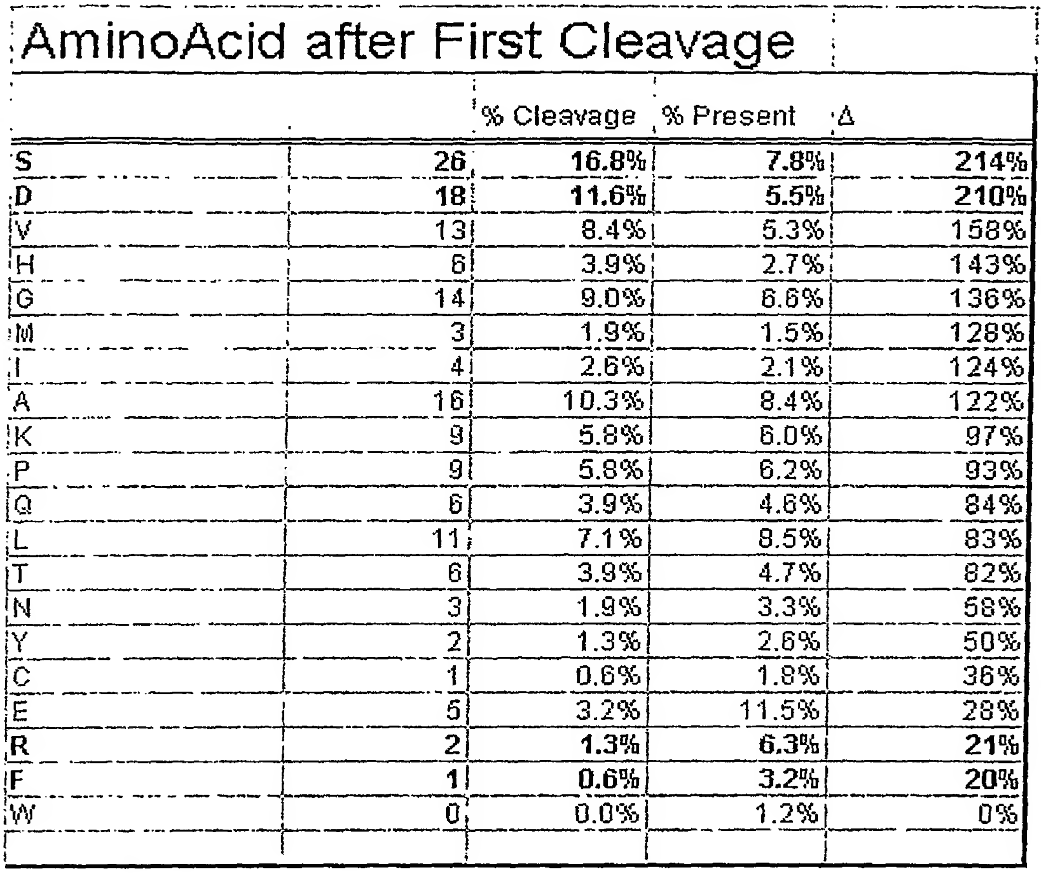
Fig. 13



AminoAcid Before First Cleavage

		% Cleavage	% Present	Δ
A	**24**	**15.5%**	**8.4%**	**184%**
R	**32**	**20.6%**	**6.3%**	**329%**
M	4	2.6%	1.5%	171%
W	3	1.9%	1.2%	168%
P	14	9.0%	6.2%	145%
N	7	4.5%	3.3%	135%
F	6	3.9%	3.2%	122%
K	10	6.5%	6.0%	107%
G	11	7.1%	6.6%	107%
L	12	7.7%	8.5%	91%
H	3	1.9%	2.7%	71%
V	5	3.2%	5.3%	61%
D	5	3.2%	5.5%	58%
Q	3	1.9%	4.6%	42%
C	1	0.6%	1.8%	36%
I	1	0.6%	2.1%	31%
T	2	1.3%	4.7%	27%
S	**3**	**1.9%**	**7.8%**	**25%**
E	**4**	**2.6%**	**11.5%**	**22%**
Y	**0**	**0.0%**	**2.6%**	**0%**
PrecursorStart	5	3.2%		

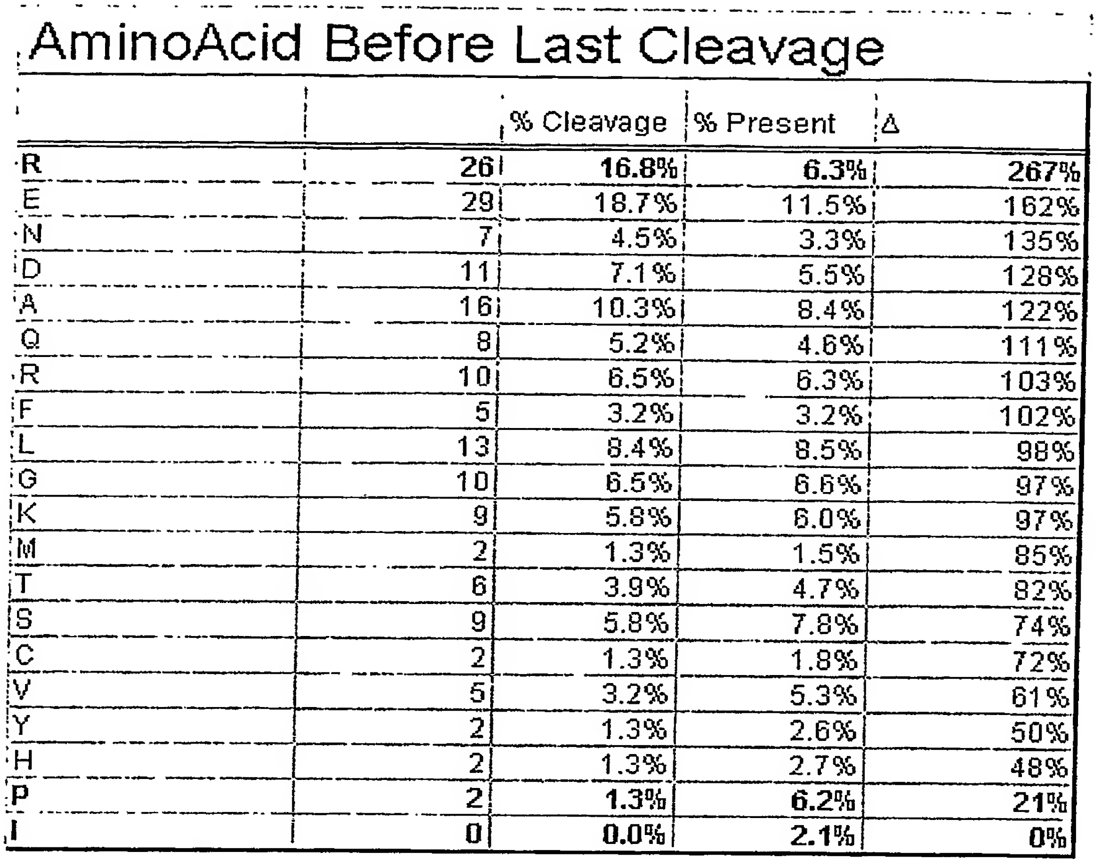
Fig. 14a



AminoAcid after First Cleavage

		% Cleavage	% Present	Δ
S	**26**	**16.8%**	**7.8%**	**214%**
D	**18**	**11.6%**	**5.5%**	**210%**
V	13	8.4%	5.3%	158%
H	6	3.9%	2.7%	143%
G	14	9.0%	6.6%	136%
M	3	1.9%	1.5%	128%
I	4	2.6%	2.1%	124%
A	16	10.3%	8.4%	122%
K	9	5.8%	6.0%	97%
P	9	5.8%	6.2%	93%
Q	6	3.9%	4.6%	84%
L	11	7.1%	8.5%	83%
T	6	3.9%	4.7%	82%
N	3	1.9%	3.3%	58%
Y	2	1.3%	2.6%	50%
C	1	0.6%	1.8%	36%
E	5	3.2%	11.5%	28%
R	**2**	**1.3%**	**6.3%**	**21%**
F	**1**	**0.6%**	**3.2%**	**20%**
W	0	0.0%	1.2%	0%

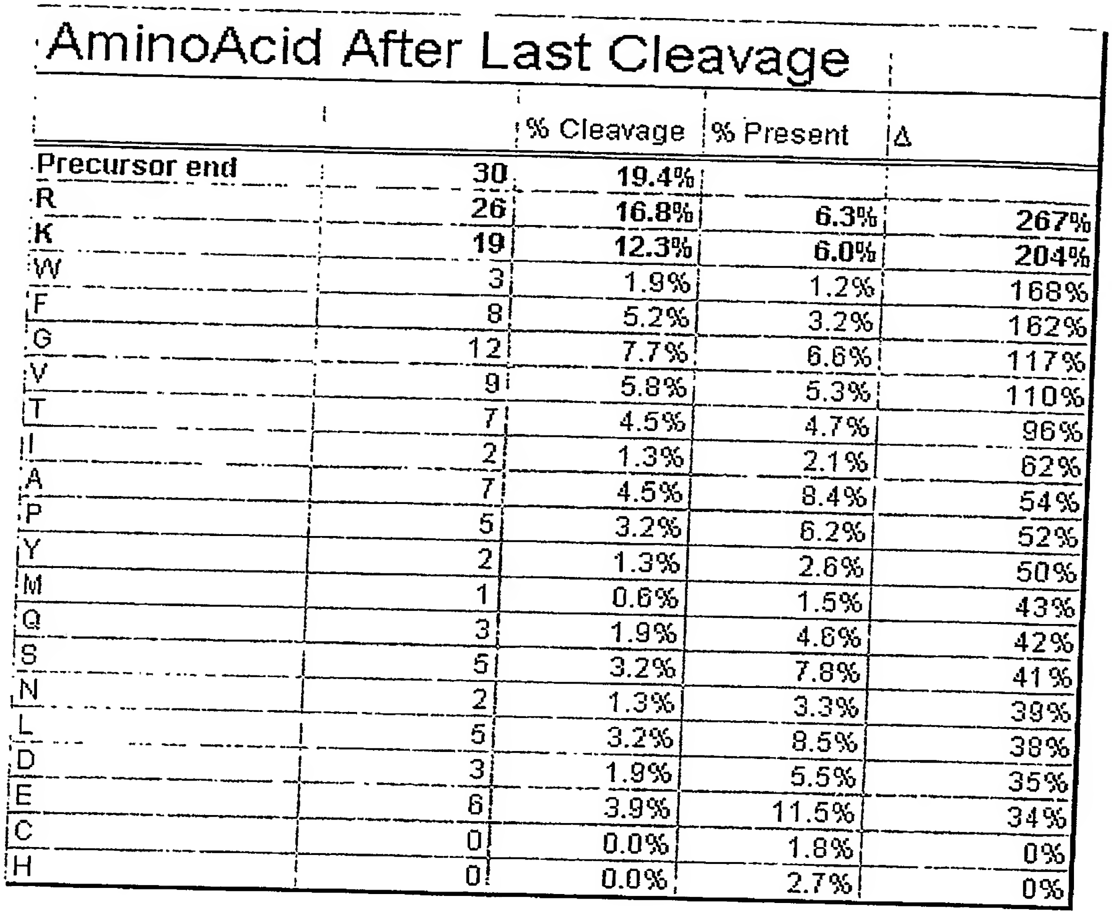
Fig. 14b



AminoAcid Before Last Cleavage

		% Cleavage	% Present	Δ
R	**26**	**16.8%**	**6.3%**	**267%**
E	29	18.7%	11.5%	162%
N	7	4.5%	3.3%	135%
D	11	7.1%	5.5%	128%
A	16	10.3%	8.4%	122%
Q	8	5.2%	4.6%	111%
R	10	6.5%	6.3%	103%
F	5	3.2%	3.2%	102%
L	13	8.4%	8.5%	98%
G	10	6.5%	6.6%	97%
K	9	5.8%	6.0%	97%
M	2	1.3%	1.5%	85%
T	6	3.9%	4.7%	82%
S	9	5.8%	7.8%	74%
C	2	1.3%	1.8%	72%
V	5	3.2%	5.3%	61%
Y	2	1.3%	2.6%	50%
H	2	1.3%	2.7%	48%
P	**2**	**1.3%**	**6.2%**	**21%**
I	**0**	**0.0%**	**2.1%**	**0%**

Fig. 14c



AminoAcid After Last Cleavage

		% Cleavage	% Present	Δ
Precursor end	**30**	**19.4%**		
R	**26**	**16.8%**	**6.3%**	**267%**
K	**19**	**12.3%**	**6.0%**	**204%**
W	3	1.9%	1.2%	168%
F	8	5.2%	3.2%	162%
G	12	7.7%	6.6%	117%
V	9	5.8%	5.3%	110%
T	7	4.5%	4.7%	96%
I	2	1.3%	2.1%	62%
A	7	4.5%	8.4%	54%
P	5	3.2%	6.2%	52%
Y	2	1.3%	2.6%	50%
M	1	0.6%	1.5%	43%
Q	3	1.9%	4.6%	42%
S	5	3.2%	7.8%	41%
N	2	1.3%	3.3%	39%
L	5	3.2%	8.5%	38%
D	3	1.9%	5.5%	35%
E	6	3.9%	11.5%	34%
C	0	0.0%	1.8%	0%
H	0	0.0%	2.7%	0%

Fig. 14d

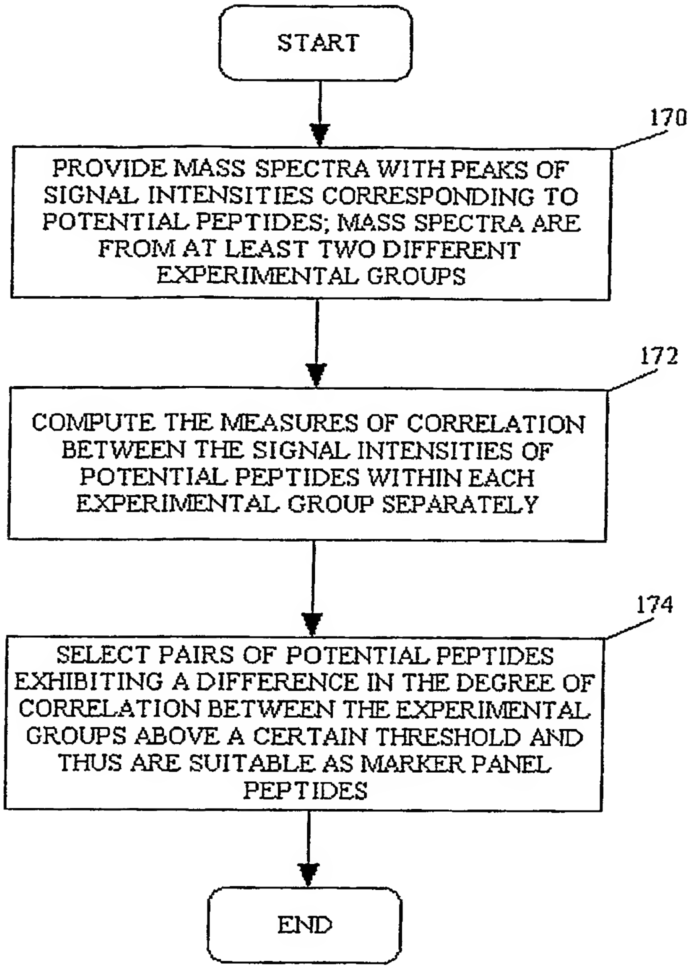

START
170
PROVIDE MASS SPECTRA WITH PEAKS OF SIGNAL INTENSITIES CORRESPONDING TO POTENTIAL PEPTIDES; MASS SPECTRA ARE FROM AT LEAST TWO DIFFERENT EXPERIMENTAL GROUPS
172
COMPUTE THE MEASURES OF CORRELATION BETWEEN THE SIGNAL INTENSITIES OF POTENTIAL PEPTIDES WITHIN EACH EXPERIMENTAL GROUP SEPARATELY
174
SELECT PAIRS OF POTENTIAL PEPTIDES EXHIBITING A DIFFERENCE IN THE DEGREE OF CORRELATION BETWEEN THE EXPERIMENTAL GROUPS ABOVE A CERTAIN THRESHOLD AND THUS ARE SUITABLE AS MARKER PANEL PEPTIDES
END

Fig. 15

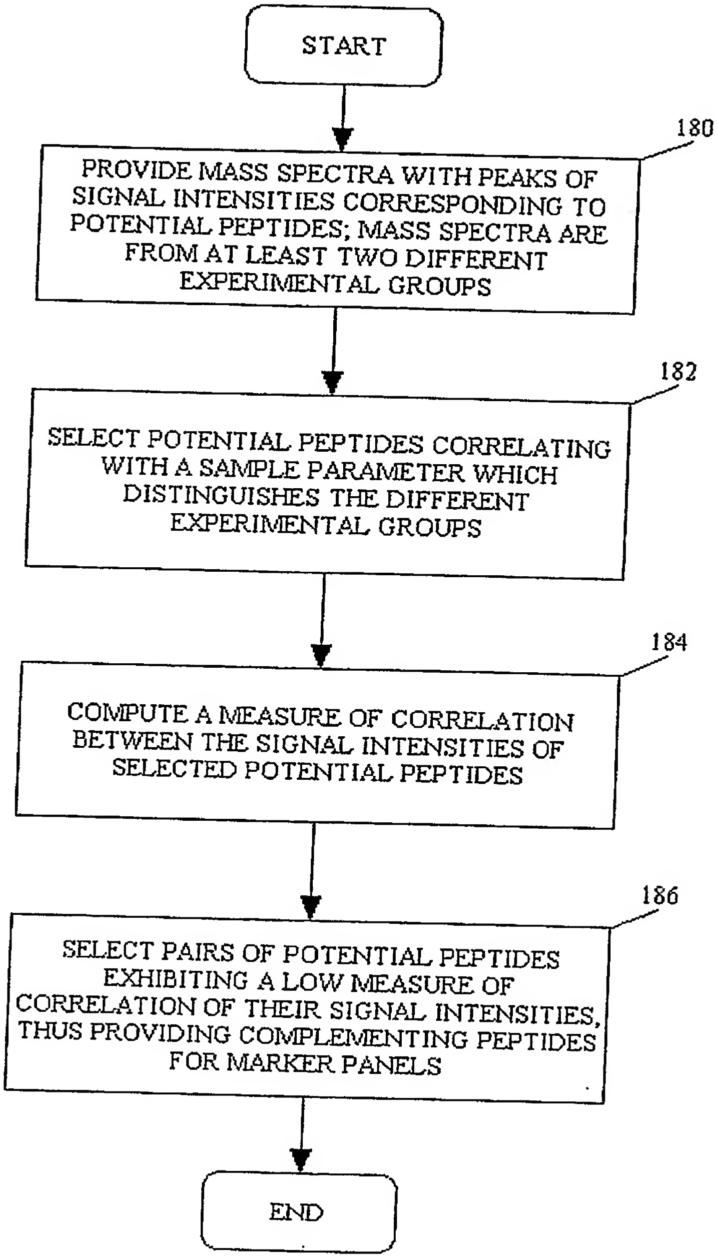

START
180
PROVIDE MASS SPECTRA WITH PEAKS OF SIGNAL INTENSITIES CORRESPONDING TO POTENTIAL PEPTIDES; MASS SPECTRA ARE FROM AT LEAST TWO DIFFERENT EXPERIMENTAL GROUPS
182
SELECT POTENTIAL PEPTIDES CORRELATING WITH A SAMPLE PARAMETER WHICH DISTINGUISHES THE DIFFERENT EXPERIMENTAL GROUPS
184
COMPUTE A MEASURE OF CORRELATION BETWEEN THE SIGNAL INTENSITIES OF SELECTED POTENTIAL PEPTIDES
186
SELECT PAIRS OF POTENTIAL PEPTIDES EXHIBITING A LOW MEASURE OF CORRELATION OF THEIR SIGNAL INTENSITIES, THUS PROVIDING COMPLEMENTING PEPTIDES FOR MARKER PANELS
END

Fig. 16

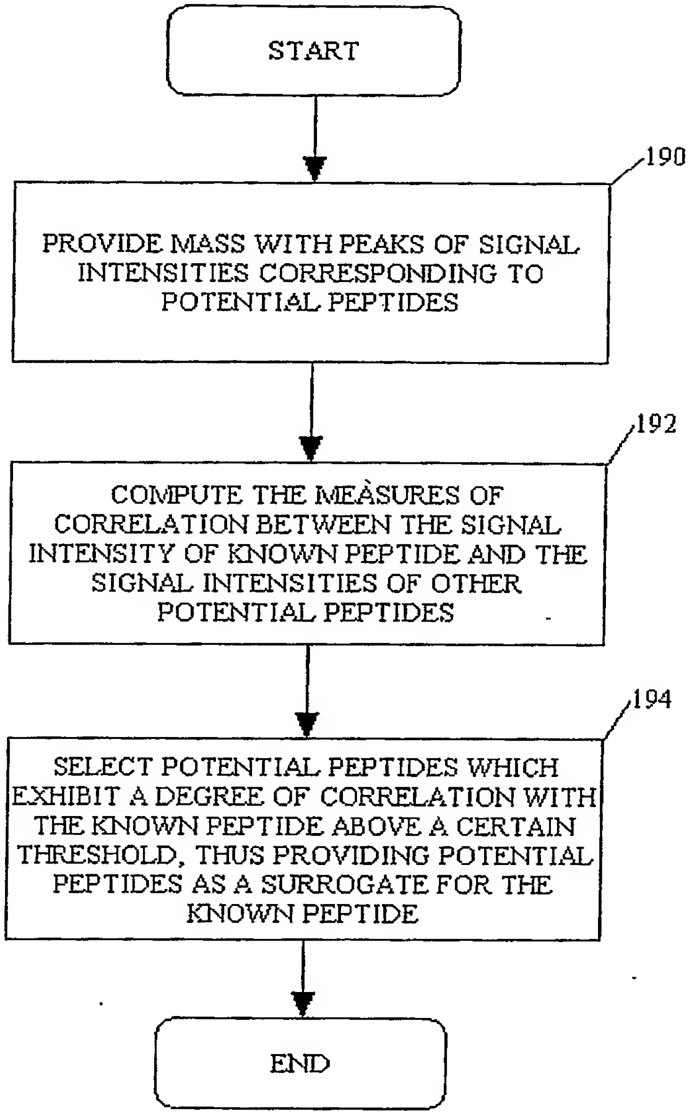

START
190
PROVIDE MASS WITH PEAKS OF SIGNAL INTENSITIES CORRESPONDING TO POTENTIAL PEPTIDES
192
COMPUTE THE MEÀSURES OF CORRELATION BETWEEN THE SIGNAL INTENSITY OF KNOWN PEPTIDE AND THE SIGNAL INTENSITIES OF OTHER POTENTIAL PEPTIDES
194
SELECT POTENTIAL PEPTIDES WHICH EXHIBIT A DEGREE OF CORRELATION WITH THE KNOWN PEPTIDE ABOVE A CERTAIN THRESHOLD, THUS PROVIDING POTENTIAL PEPTIDES AS A SURROGATE FOR THE KNOWN PEPTIDE
END

Fig. 17

Case No.	Fraction 54 m/z 2743.0	Fraction 54 m/z 1371.5	Fraction 56 m/z 2927.2	....	Fraction 20 m/z 11143	....
	k=20	k=19	k=16		k=19	
1	21648	712	2620		1452	
2	1830	1320	1199		2554	
3	21353	2022	3159		2139	
4	24223	2454	2431		2169	
5	3725	2719	982		1038	
6	13548	2956	1689		1300	
7	16606	3260	1402		2213	
8	20902	3435	624		887	
9	10321	3444	427		2145	
10	31047	3498	2238		1516	
11	31142	3592	4405		1255	
12	37241	3745	2785		1739	
13	22656	3822	2264		2576	
14	24366	3852	1752		1139	
15	16638	3935	1147		2182	
16	37171	4092	2393		1069	
17	33188	4115	1578		1681	
18	27596	4127	3228		1463	
19	39668	4348	3604		318	
20	12983	4362	1048		3039	
21	14420	4488	899		2676	
22	23261	4634	1965		3071	
23	30507	4710	2715		1247	
24	41494	4928	5343		1238	
25	36664	5107	3914		3156	
26	42465	5135	2729		1768	
27	42551	5135	3010		2500	
28	35473	5201	2242		1978	
29	48611	5906	2381		1075	
30	28413	5914	1855		3189	
31	35258	5954	3368		2140	
32	44774	6056	4167		670	
33	46137	6465	7640		1719	
34	40892	6531	1630		1241	
35	48202	7076	11222		3826	
36	43760	7183	4771		1565	
37	50211	7316	5443		2060	

Fig. 18a

Case No.	Fraction 54 m/z 2743.0	Fraction 54 m/z 1371.5	Fraction 56 m/z 2927.2	...	Fraction 20 m/z 1114.3	...
38	49824	7410	3004		1113	
39	50785	7752	6412		1616	
40	46200	7821	3689		3725	
41	52471	7949	5395		1837	
42	49299	8280	4623		1207	
43	45032	8483	4881		1566	
44	51224	8562	6481		2194	
45	51901	8638	10081		2047	
46	51084	8776	14193		1478	
47	50928	8852	6635		287	
48	50707	10097	8877		1458	
49	52304	10259	6244		1860	
50	48355	10661	5195		3695	
51	51363	10685	11403		1261	
52	54423	10846	11299		2067	
53	55167	11041	12868		1545	
54	55091	11539	5597		2381	
55	56825	11912	7718		2409	
56	53173	12022	8865		1969	
57	51649	12057	7855		1295	
58	51328	12095	9035		2043	
59	53464	12641	6408		856	
60	54542	12891	10363		1858	
61	56950	13172	7586		1802	
62	43273	14559	20080		596	
63	57335	14922	12288		2916	
64	55118	14997	10078		1761	
65	57147	16164	7726		2626	
66	55584	16216	17106		2623	
67	59414	16550	15122		539	
68	57093	16689	19689		2078	
69	57841	18254	16079		1659	
70	54084	18734	19524		395	
71	56325	22730	10828		2326	
72	58386	24159	16681		1631	
73	54843	26671	44356		3183	
74	53935	27937	30189		1403	

Fig. 18b

	Fraction 54 m/z 2743.0 and ...		
Measure of Association	Fraction 54 m/z 1371.5	Fraction 56 m/ 2927.2	Fraction 20 m/z 1114.3
Spearman's rank order correlation	0.9298	0.8761	-0.0044
Pearson's product moment correlation	0.7318	0.5855	-0.0781
Kendall's rank correlation tau	0.7704	0.6919	0.0107
MST diameter	50	40	29

Fig. 19

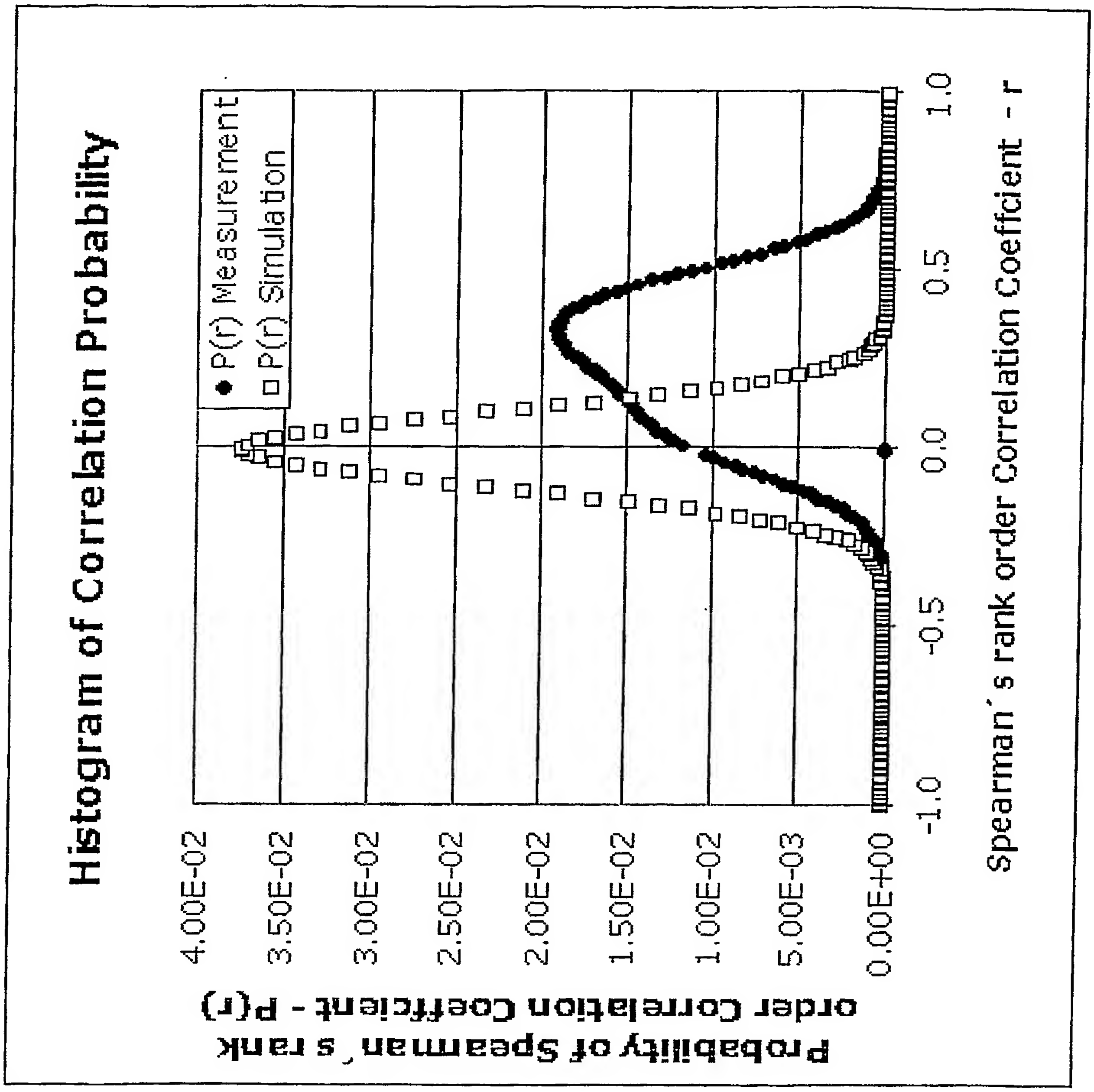

Histogram of Correlation Probability
● P(r) Measurement
□ P(r) Simulation
4.00E-02
3.50E-02
3.00E-02
2.50E-02
2.00E-02
1.50E-02
1.00E-02
5.00E-03
0.00E+00
-1.0
-0.5
0.0
0.5
1.0
Spearman´s rank order Correlation Coeffcient - r
Probability of Spearman´s rank order Correlation Coefficient - P(r)

Fig. 20

Hub-Peptide	Correlation	Related peptide	Relative Mono-isotopic mass [Da]	Amino Acid Sequence
Chromogranin A 97 - 131			3905.764	HSGF EDELSEVLEN QSSQAELKEA VEEPSSKDVME
	r=0.67	Secretogranin I 88-132	4605.025	DPADASEAHESSSRGEAGAP GEEDIQGPTKADTEKWAEGG GHSRE
	r=0.71	Secretogranin II 529-566	4152.921	G QGSSEDDLQEE EQIEQAIKEH LNQGSSQETD KLAPVS
	r=0.72	Secretogranin V 181-202	2448.334	SVNPYLQGQRLDNVVAKKSV PH

Fig. 21

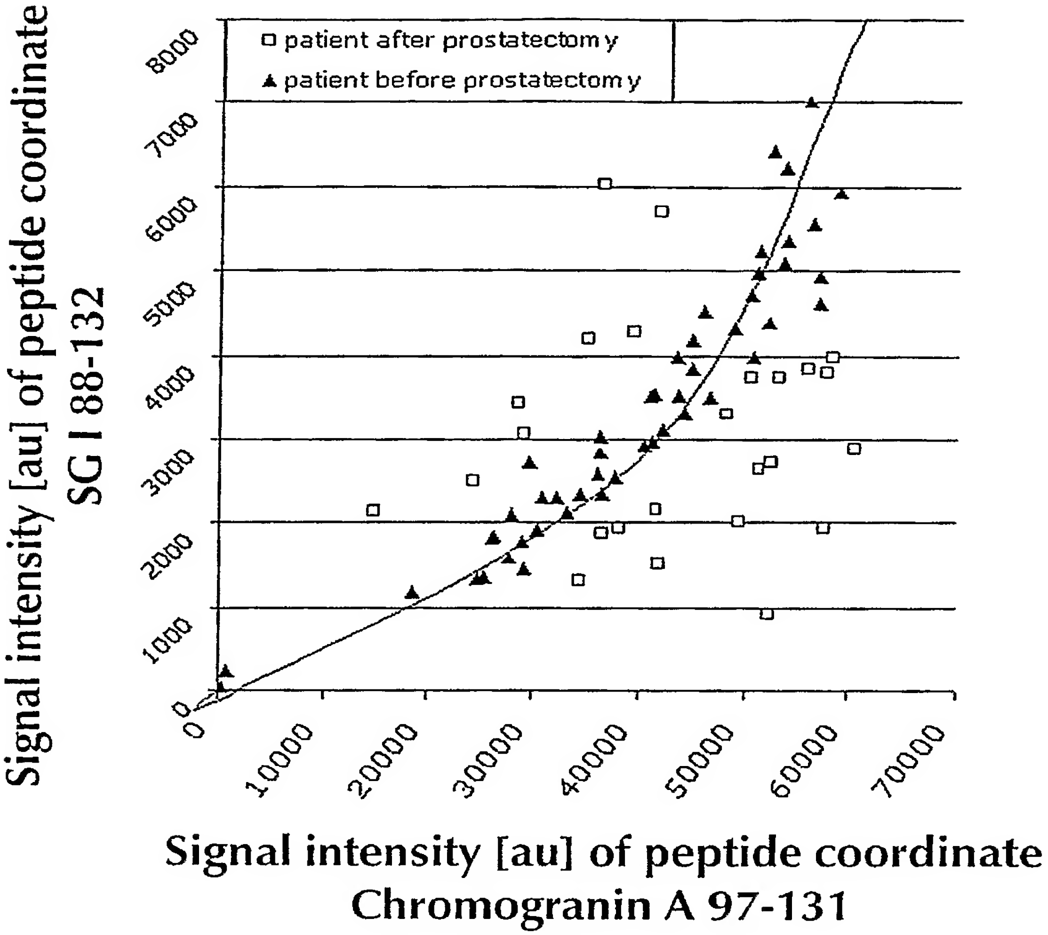

□ patient after prostatectomy
▲ patient before prostatectomy
Signal intensity [au] of peptide coordinate SG I 88-132
0
1000
2000
3000
4000
5000
6000
7000
8000
0
10000
20000
30000
40000
50000
60000
70000
Signal intensity [au] of peptide coordinate Chromogranin A 97-131

Fig. 22

	Fraction 54 m/z ~~2743.0~~	Fraction 54 m/z 1371 5	Fraction 56 m/z 2927.2	....	Fraction 20 m/z 1114.3	....
	~~k=0~~	k=1	k=2		k=19	
case 1	~~21648~~	3747	7051		1452	
case 2	~~1830~~	4355	5630		2554	
case 3	~~21353~~	5057	7590		2139	
case 4	~~24223~~	5489	6862		2169	
case 5	~~3725~~	5754	5413		1038	
case 6	~~13548~~	5991	6120		1300	
case 7	~~16686~~	6295	5833		2213	
case 8	~~20902~~	6470	5055		887	
case 9	~~10321~~	6479	4858		2145	
case 10	~~31047~~	6533	6669		1516	
case 11	~~31142~~	6627	8835		1255	
case 12	~~37241~~	6780	7216		1739	
case 13	~~22656~~	6857	6695		2576	
case 14	~~24366~~	6887	6183		1139	
case 15	~~16638~~	6970	5578		2182	
case 16	~~37171~~	7127	6824		1069	
case 17	~~33188~~	7150	6009		1681	
case 18	~~27596~~	7162	7659		1463	
case 19	~~39668~~	7383	8035		318	
case 20	~~12983~~	7397	5479		3039	
case 21	~~14420~~	7523	5330		2676	
case 22	~~23261~~	7669	6396		3071	
case 23	~~30587~~	7745	7146		1247	
case 24	~~41494~~	7963	9774		1238	
case 25	~~36664~~	8142	8345		3156	
case 26	~~42465~~	8170	7160		1768	
case 27	~~42551~~	8170	7441		2500	
case 28	~~35473~~	8236	6673		1978	
case 29	~~48611~~	8941	6812		1075	
case 30	~~28413~~	8949	6286		3189	
case 31	~~35258~~	8989	7799		2140	
case 32	~~44774~~	9091	8598		670	
case 33	~~46137~~	9500	12071		1719	
case 34	~~40892~~	9566	6061		1241	
case 35	~~48202~~	10111	15653		3826	
case 36	~~43760~~	10218	9202		1565	

Fig. 23a

	Fraction 54 m/z 2743.0	Fraction 54 m/z 1371.5	Fraction 56 m/z 2927.2	....	Fraction 20 m/z 1114.3	....
case 37	~~58211~~	10351	9874		1113	
case 38	~~49704~~	10445	7435		1616	
case 39	~~50785~~	10787	10843		3725	
case 40	~~46230~~	10856	8120		1837	
case 41	~~52471~~	10984	9826		1207	
case 42	~~49299~~	11315	9054		1566	
case 43	~~45032~~	11518	9312		2194	
case 44	~~51224~~	11597	10912		2047	
case 45	~~51901~~	11673	14512		1478	
case 46	~~51084~~	11811	18624		287	
case 47	~~50928~~	11887	11066		1458	
case 48	~~50707~~	13132	13308		1860	
case 49	~~52204~~	13294	10675		3695	
case 50	~~48355~~	13696	9626		1261	
case 51	~~51363~~	13720	15834		2067	
case 52	~~54423~~	13881	15730		1545	
case 53	~~55167~~	14076	17299		2381	
case 54	~~55091~~	14574	10028		2409	
case 55	~~56825~~	14947	12149		1969	
case 56	~~53173~~	15057	13296		1295	
case 57	~~51649~~	15092	12286		2043	
case 58	~~51328~~	15130	13466		856	
case 59	~~53464~~	15676	10839		1858	
case 60	~~54542~~	15926	14794		1802	
case 61	~~56950~~	16207	12017		596	
case 62	~~43273~~	17594	24511		2916	
case 63	~~57335~~	17957	16719		1761	
case 64	~~55118~~	18032	14509		2626	
case 65	~~57147~~	19199	12157		2623	
case 66	~~55584~~	19251	21537		539	
case 67	~~59414~~	19585	19553		2078	
case 68	~~57093~~	19724	24120		1659	
case 69	~~57841~~	21289	20510		395	
case 70	~~54084~~	21769	23955		2326	
case 71	~~56325~~	25765	15259		1631	
case 72	~~58386~~	27194	21112		3183	
case 73	~~54843~~	29706	48787		1403	
case 74	~~53935~~	30972	34620		1113	

Fig. 23b

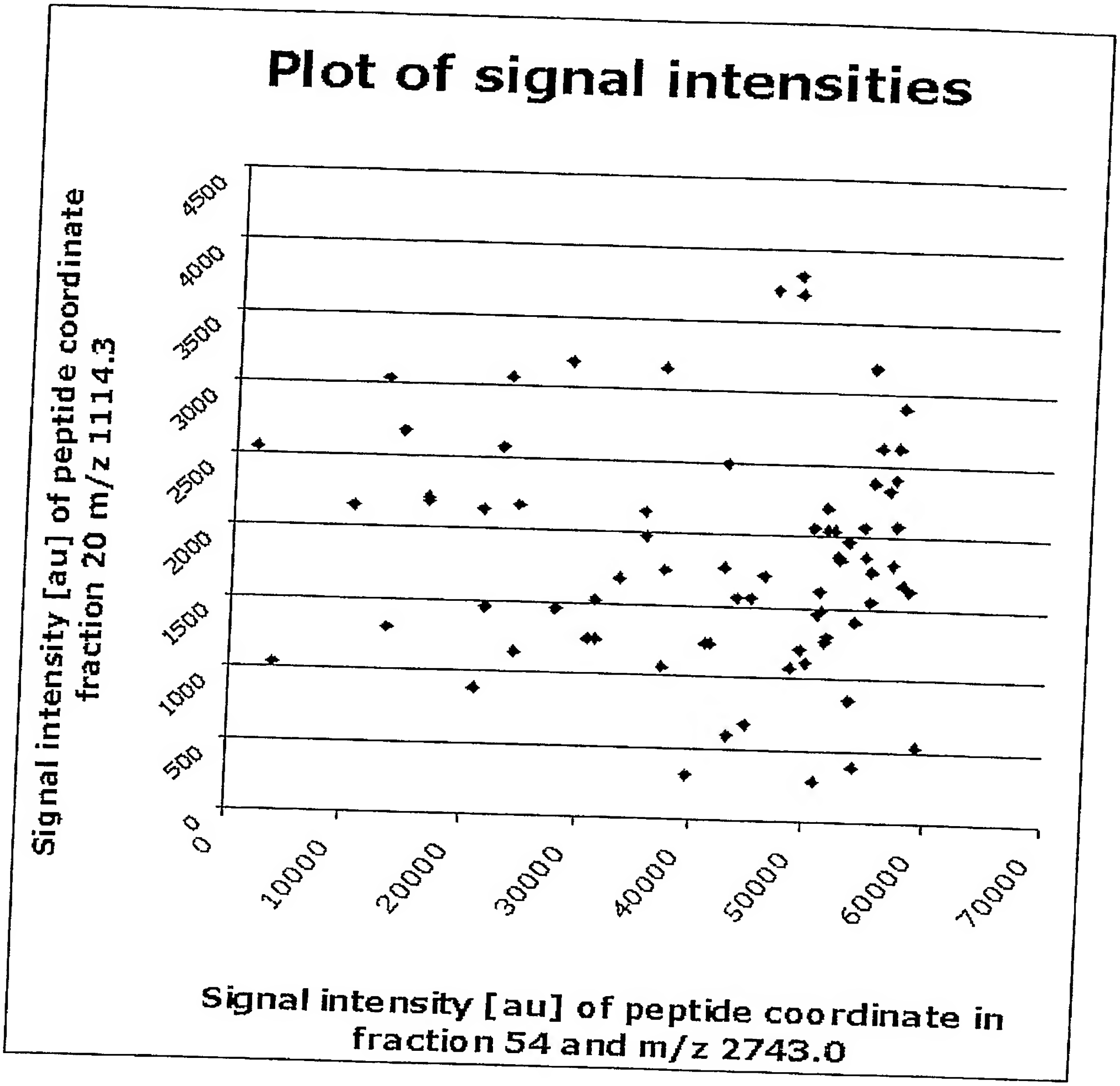

Plot of signal intensities
Signal intensity [au] of peptide coordinate fraction 20 m/z 1114.3
0
500
1000
1500
2000
2500
3000
3500
4000
4500
0
10000
20000
30000
40000
50000
60000
70000
Signal intensity [au] of peptide coordinate in fraction 54 and m/z 2743.0

Fig. 24a

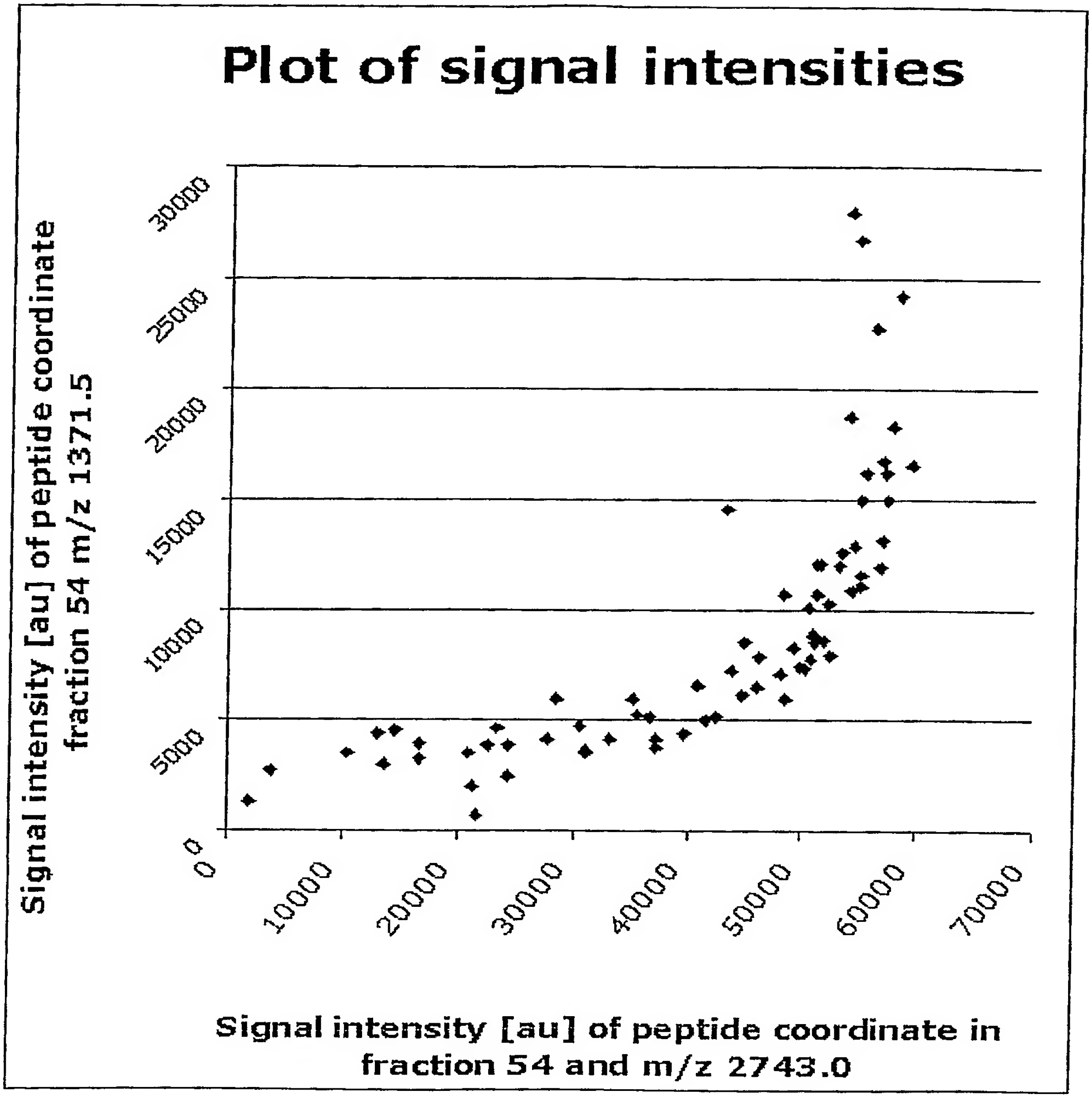

Plot of signal intensities
Signal intensity [au] of peptide coordinate fraction 54 m/z 1371.5
Signal intensity [au] of peptide coordinate in fraction 54 and m/z 2743.0
0
5000
10000
15000
20000
25000
30000
0
10000
20000
30000
40000
50000
60000
70000

Fig. 24b

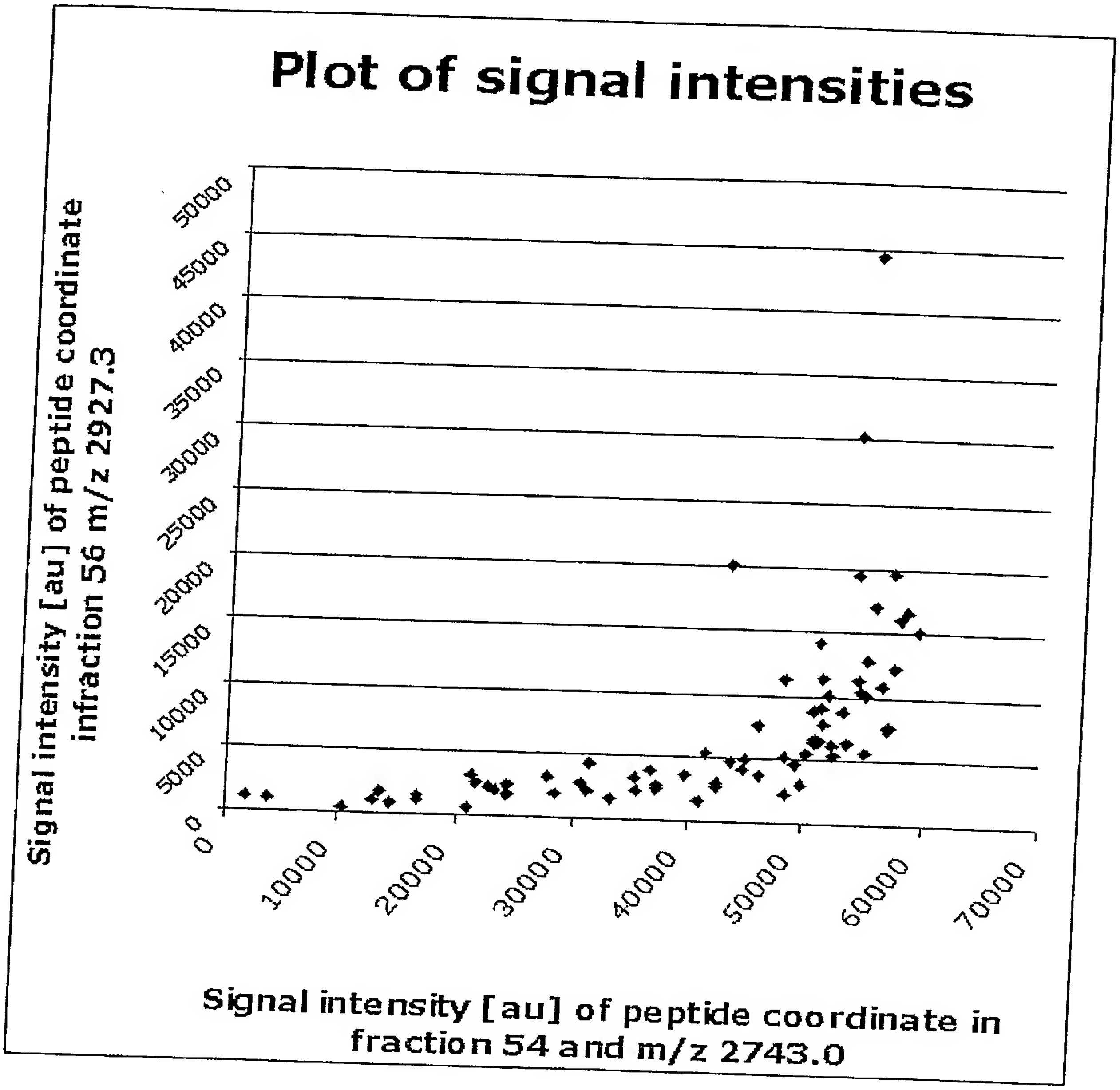

Plot of signal intensities
Signal intensity [au] of peptide coordinate infraction 56 m/z 2927.3
0
5000
10000
15000
20000
25000
30000
35000
40000
45000
50000
0
10000
20000
30000
40000
50000
60000
70000
Signal intensity [au] of peptide coordinate in fraction 54 and m/z 2743.0

Fig. 24c

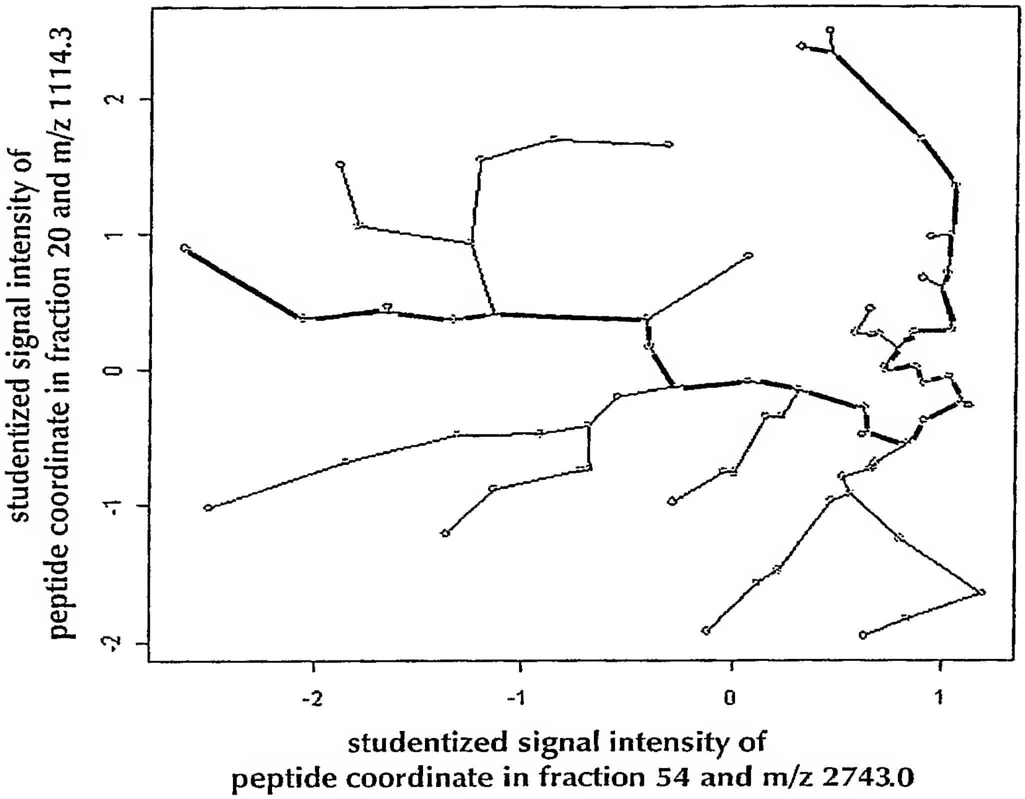

studentized signal intensity of
peptide coordinate in fraction 20 and m/z 1114.3
-2
-1
0
1
2
-2
-1
0
1
studentized signal intensity of
peptide coordinate in fraction 54 and m/z 2743.0

Fig. 25a

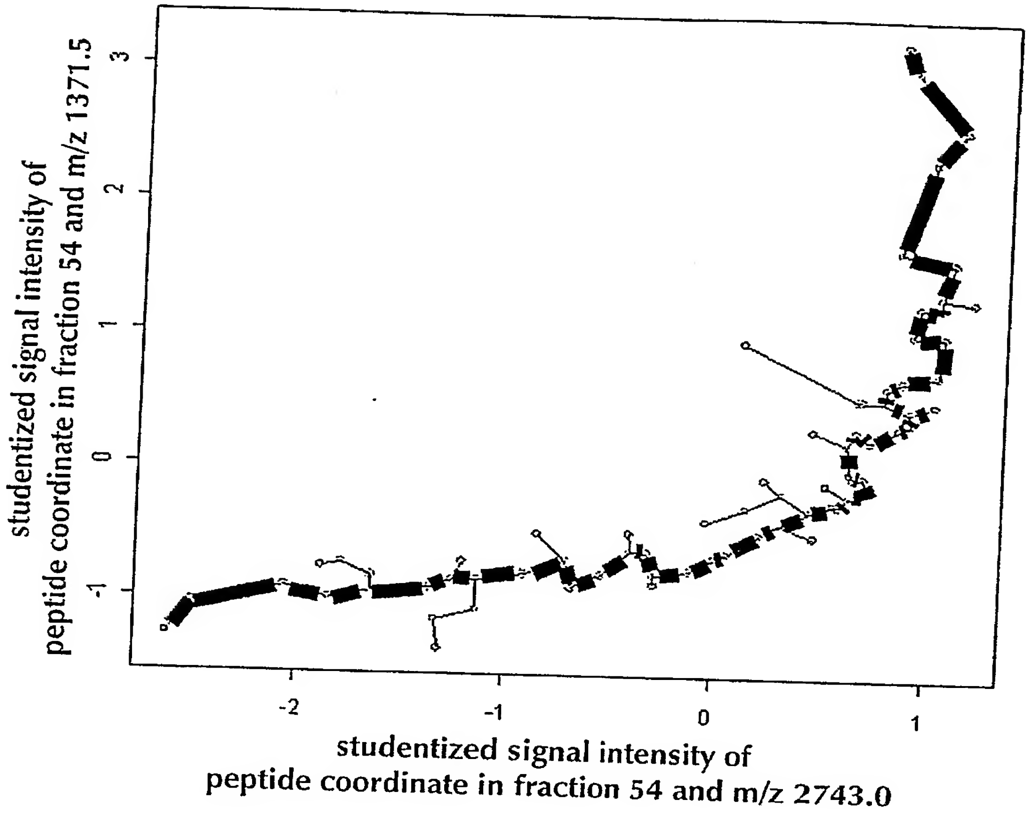

studentized signal intensity of
peptide coordinate in fraction 54 and m/z 1371.5
-1
0
1
2
3
-2
-1
0
1
studentized signal intensity of
peptide coordinate in fraction 54 and m/z 2743.0

Fig. 25b

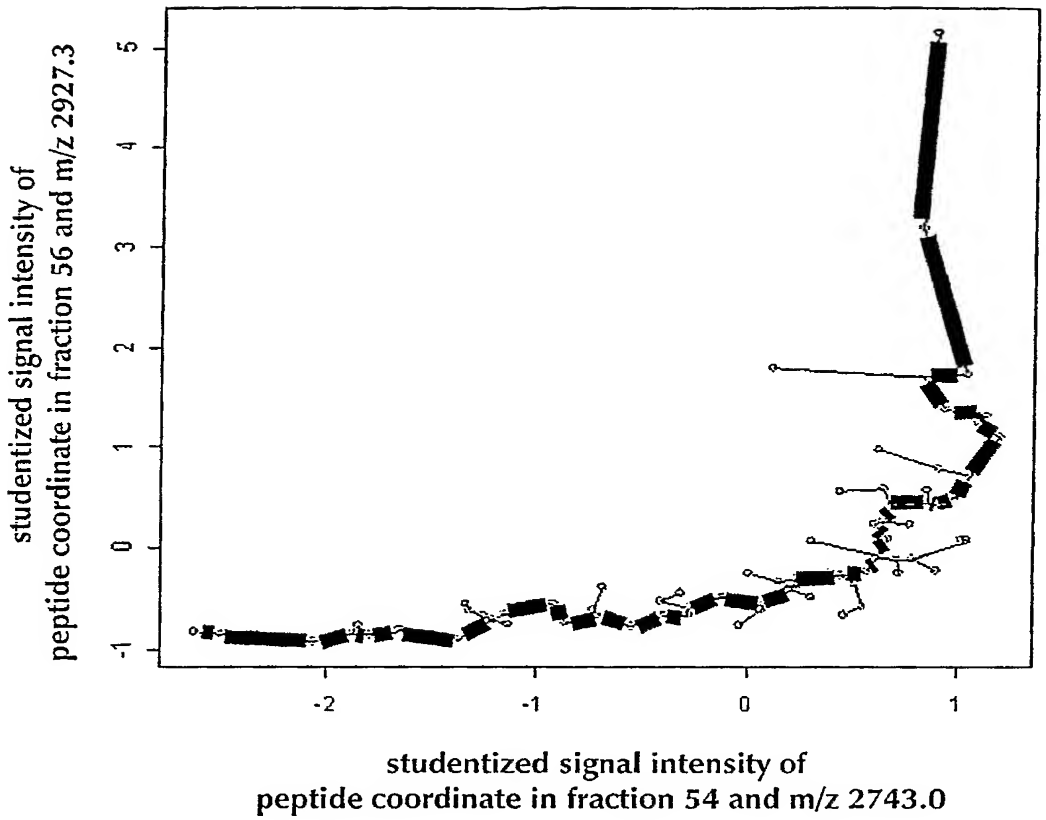

studentized signal intensity of
peptide coordinate in fraction 56 and m/z 2927.3
5
4
3
2
1
0
-1
-2
-1
0
1
studentized signal intensity of
peptide coordinate in fraction 54 and m/z 2743.0

Fig. 25c

	n			
m/z	1	2	3	4
1371.5	-1371.5	0.0	1371.5	2743.0
2927.3	183.3	3109.6	6035.9	8962.2

Fig. 26

```
1         10        20        30        40
|         |         |         |         |
MKWVTFISLL FLFSSAYSRG VFRRDAHKSE VAHRTYDLGE

          50        60        70        80
          |         |         |         |
ENFKALVLIA KREAQYLQQC PFEDHVKLVN EVTEFAKTCV

          90        100       110       120
          |         |         |         |
ADESAENCDK SLHTLFGDKL CTVATLRETY GEMADCCAKQ

          130       140       150       160
          |         |         |         |
EPERNECFLQ HKDDNPNLPR LVRPEVDVMC TAFHDNEETF

          170       180       190       200
          |         |         |         |
LKKYLYEIAR RHPYFYAPEL LFFAKRYKAA FTECCQAADK

          210       220       230     238
          |         |         |       |
AACLLPKLDE LRDEGKASSA KQRLKCASLQ KFGERAFK
```

Fig. 27